Molecular Cloning and Characterization of Nitrate Reductase from *Ricinus communis* L. Heterologously Expressed in *Pichia pastoris*

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Thanks and dedication to

My parents……...	*without them I could not be here*
Buddha Shakyamuni…..…	*without him I would never appreciate the truth*

Summary

Background: In a previous study, nitrate reductase (NR, EC 1.6.6.1) from leaves of *Ricinus communis* L. showed different regulatory properties from most other higher plants NR's by an unusually strong Mg^{2+}-sensitivity, a different pH-activity profile and only little ATP-dependent inactivation.

The aim of this work was to elucidate the deviating properties of Ricinus NR in more details, from both molecular and physiological aspects. For that purpose, the NR gene from *R. communis* was cloned, expressed heterologously and characterized.

Results: The deduced protein sequence showed that Ricinus NR shared high similarity with other NRs, apart from the N-terminal region. In the N-terminal region, the Ricinus NR possesses an acidic stretch which is conserved only in higher plants. Within the Moco-binding domain the Ricinus NR contained few amino acid residues which were unique in comparison with 17 plant NRs, including His103, Gln123, Val266 and Ala284 where other NRs possess asparagine, arginine, aspartate and proline.

In the Dimer interface and Hinge 1 regions, the Ricinus NR also had some unique residues like Asn460 and Ala498 where other NRs have isoleucine and glycine instead. The Ricinus NR possesses an Arg482 which provides an additional predicted Trypsin cleavage site within ^{481}KRHK^{484} (while most of plant-NRs possess KPHK). Additionally, the Ricinus NR contains a serine phosphorylation site (Ser-526) within the potential 14-3-3 binding motif ^{523}KSVS*TP^{528}, which is a common characteristic of nitrate reductases. In the C-Terminus of Ricinus NR a sequence ^{886}CGPPP^{890} confirmed that Ricinus NR is a NADH-specific enzyme.

Functional Ricinus NR protein was expressed in *Pichia pastoris* and compared with the features of Arabidopsis NR2 synthesized by the same expression system (AtNR2). The recombinant Ricinus NR (RcNR) itself was unresponsive to the incubation with MgATP, and so was AtNR2. As yeast extracts might lack factors required for NR regulation, desalted leaf extracts containing NR kinases and 14-3-3s were prepared from 4-day darkened (and therefore NR-free) leaves of Arabidopsis (ADL), spinach (SDL) and Ricinus (RDL), and added to the assay of RcNR and AtNR2 to check for

ATP-dependent inactivation and Mg^{2+}-sensitivity. When RcNR was combined with the NR-free extracts described above, it's unusually high Mg^{2+}-sensitivity was restored only by incubation with RDL, but it remained unresponsive to ATP. In contrast, AtNR2 became inactive when incubated with the protein mixtures and ATP. It is obvious that one or some factors existing in RDL could interact with RcNR and therefore provide its high Mg^{2+}-sensitivity. Interestingly, incubation of AtNR2 with different NR-free leaf extracts gave a significant activation of the enzyme activities, both in Mg^{2+} and EDTA, which were not observed in the case of RcNR. Moreover, using ammonium sulfate to fractionation the RDL revealed that about 0.2 mg of the protein factor(s) from 0-35% of ammonium sulfate precipitation was sufficient to provide the maximum inhibition of the RcNR.

Conclusions: The insensitivity to ATP appears an inherent property of Ricinus NR, whereas the high Mg^{2+}-sensitivity depends on one or several factors in Ricinus leaves. This as yet unknown factor(s) was boiling-sensitive and could be precipitated by ammonium sulfate. It appeared to interact specifically with recombinant Ricinus-NR to provide the Mg^{2+}-sensitivity of the authentic leaf enzyme. Presumably, there is also a positive regulatory factor(s) for nitrate reductase existing in the leaves of higher plants.

Zusammenfassung

Hintergrund: In einer vorhergehenden Studie wurde gezeigt, dass die Nitratreduktase (NR, EC 1.6.6.1) aus Blättern von *Ricinus communis* L. im Vergleich zu NRs der meisten anderen höheren Pflanzen durch verschiedene Faktoren unterschiedlich reguliert wird. Die Aktivität ist ungewöhnlich Mg^{2+}-sensitiv, zeigt ein verändertes pH-Profil und ist nur gering ATP-abhängig inaktivierbar.

Das Ziel dieser Arbeit war, die abweichenden Eigenschaften von Ricinus NR, aus molekularer und physiologischer Sicht detaillierter aufzuklären. Zu diesem Zweck wurde das NR Gen von *R. communis* geklont, heterolog exprimiert und charakterisiert.

Ergebnisse: Die abgeleitete Proteinsequenz zeigte, dass Ricinus NR hohe Ähnlichkeit mit anderem NRs teilte, abgesehen von der N-terminalen Region. In der N-terminalen Region besitzt die Ricinus NR eine säurehaltige Sequenz, die nur in den höheren Pflanzen konserviert ist. In der Moco-bindenden Region waren einige in 17 Pflanzen NRs konservierte Aminosäurepositionen verändert. Zu diesen Positionen gehörten His103, Gln123, Val266 und Ala284, die Asparagin, Arginin, Aspartat und Prolin in den anderen Pflanzen ersetzten. Auch an der Dimerisierungs- und Hinge 1-Region, zeigte die Ricinus NR eine veränderte Aminosäuresequenz. Anstatt Isoleucin und Glycin, besaß die Ricinus NR an den Stellen 460 und 498 Asparagin und Alanin. Durch ein Arg an der Stelle 482 kommt es zu einer zusätzliche Trypsinschnittstelle innerhalb des ^{481}KRHK^{484}-Motivs (die meisten NR besitzen hier KPHK).
Zusätzlich enthält die Ricinus NR eine Serinphosphorylierungsstelle (Ser-526) innerhalb des möglichen 14-3-3 Bindemotivs ^{523}KSVS*TP^{528}, was eine allgemeine Eigenschaft von Nitratreduktasen ist. Im C-Terminus von Ricinus NR bestätigte die Sequenz ^{886}CGPPP^{890}, dass die Ricinus NR ein NADH-spezifisches Enzym ist.

Die Ricinus NR und Arabidopsis NR2 (AtNR2) wurden in *Pichia pastoris* funktionell exprimiert und die Eigenschaften miteinander verglichen. Die rekombinante Ricinus NR (RcNR) selbst wurde nicht durch die Inkubation mit MgATP inhibiert, ebenso AtNR2. Da der Hefeextrakt vermutlich die Faktoren zur Regulierung der NR nicht enthält, wurden entsalzte Blattextrakte von Arabidopsis (ADL), Spinat (SDL) und

Ricinus (RDL) zugesetzt, die Kinasen und 14-3-3 Proteine enthielten. Damit keine endogenen NRs sich im Extrakt befinden wurden die Blätter vor Extraktion 4 Tage im Dunkeln gehalten.

In Bezug auf die Inhibierung der NR durch ATP wurde festgestellt, dass die RcNR gegenüber einer solchen Inhibierung unempfindlich ist, AtNR2 dagegen in jedem Fall durch ATP inaktiviert wird. Bei Kombination von RcNR mit NR-freien Extrakten aus Pflanzen zeigte sich die erhöhte Mg^{2+}-Sensitivität nur, wenn man RcNR mit RDL inkubierte, nicht aber wenn man RcNR mit SDL oder ADL inkubierte. Es liegt auf der Hand, dass ein oder einige Faktoren in RDL vorkommen, die mit RcNR interagieren und seine hohe Mg^{2+}-Sensitivität hervorrufen.

Außerdem, ergab eine Inkubation von AtNR2 mit unterschiedlichen NR-freien Blattextrakten eine bedeutende Aktivierung der Enzymaktivitäten, sowohl in Anwesenheit von Mg^{2+} als auch EDTA. Dies wurde jedoch nicht für die RcNR festgestellt. Nach Verwendung von Ammoniumsulfat zur Fraktionierung des RDL, fand man zusätzlich heraus, dass ungefähr 0,2 mg des Proteins der Fraktion die mit 0-35% Ammoniumsulfat gefällt wurde ausreichten die maximale Hemmung des RcNR hervorzurufen.

Schlussfolgerungen: Die Unempfindlichkeit gegenüber ATP erscheint eine angeborene Eigenschaft von Ricinus NR, während die hohe Mg^{2+}-Sensitivität von einem oder einigen Faktoren in den Blättern von Ricinus abhängt. Diese(r) bis jetzt unbekannte Faktor(en) war Hitze-sensitiv und konnte durch Ammoniumsulfat ausgefällt werden. Er scheint spezifisch auf die rekombinante Ricinus-NR einzuwirken, und liefert eine Mg^{2+}-Sensitivität vergleichbar dem authentischen Blattenzym. Außerdem gibt es vermutlich auch positiv regulierende Faktor(en) für die Nitratreduktase aus Blättern höherer Pflanzen.

1. Introduction

1.1 Overview of nitrate assimilation in higher plants

Nitrogen is one of the most important mineral elements that plants require. There are two routes of biological N acquisition: nitrate assimilation and nitrogen fixation. The nitrate assimilation pathway is the main entry for inorganic nitrogen which is later incorporated into organic compounds. Before entering the assimilation pathway, nitrate has to be taken up by the cell. Following its uptake from the soil by plant roots, nitrate is reduced to ammonium prior to assimilation into organic compounds.

The first step of this process is a two-electron reduction of nitrate to nitrite, catalyzed by nitrate reductase (NR; EC 1.6.6.1-3).

$$\mathbf{NO_3^- + NAD(P)H + H^+ + 2e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O}$$

The second step is a six-electron reduction of nitrite to ammonium, catalyzed by nitrite reductase (NiR; EC 1.7.7.1).

$$\mathbf{NO_2^- + 6Fd_{red} + 8H^+ + 6e^- \rightarrow NH_4^+ + 6Fd_{ox} + 2H_2O}$$

Subsequently, ammonium is incorporated into amino acids, catalysed primarily by glutamine synthetase (GS) and glutamate synthase (GOGAT) (for reviews, see Crawford, 1995; Lam et al., 1996; Stitt, 1999).

1.2 Overview of nitrate reductase

Nitrate reductase is a complex enzyme containing several prosthetic groups. Recent data on 3D structure of NR (Lu et al., 1994; Campbell, 1996), once thought to contain only 3 domains, indicate that it actually contains 5 structurally distinct domains. From the N-terminus to the C-terminus, these are: molybdenum cofactor (Moco), dimer interface, cytochrome b (Cb), FAD and NADH (Figure 1.1). When the FAD and NADH domains are combined, the cytochrome b reductase fragment (CbR) is formed. If the Cb domain is connected to CbR, it is called the cytochrome c reductase fragment (CcR). On the other hand, there are three sequence regions with no similarity to another protein and varying in sequence among NRs. Those are the N-terminal regions, which are rich in acidic residues; Hinge 1, which contains a phosphorylation site and a trypsin proteolytic site; Hinge 2, which also contains a protease site.

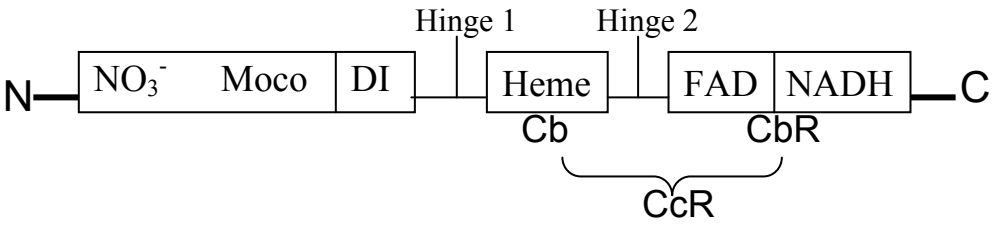

Figure 1.1 Sequence model of the enzyme. DI, dimer interface. (Campbell, 1999; modified)

The native enzyme is a homodimer or a homotetramer (in case of *Chlorella*) (Howard and Solomonson, 1982). In higher plants, the size of a monomer is ranging from 100 to 115 kDa. The intracellular location of NR is still discussed, but most evidence points to a cytosolic localization (for a review, see Solomonson and Barber, 1990).

The first higher plant NR cloned was from barley (Cheng et al., 1986) and later a number of *Nia* sequences (including cDNA and genomic DNA) have been identified from different organisms. Three forms of nitrate reductase have been found in eukaryotes. The most common form in higher plants is a NADH-specific NR (EC 1.6.6.1). A NAD(P)H-bispecific NR (EC 1.6.6.2) has been identified in several plants, existing as the sole isoform in *Betula pendula* (Friemann et al., 1991) or a second isoform along with the NADH-specific NR as in soybean and monocot species including maize, rice and barley (Kleinhofs and Warner, 1990). The third form is NADPH-specific (EC 1.6.6.3) which is present in fungi and in mosses.

In addition to catalyze the formation of nitrite from nitrate, NR also has a secondary function in that it catalyses the production of nitric oxide (NO) from nitrite, and of molecular oxygen to superoxide (Dean and Harper, 1988; Yamasaki and Sakihama, 2000). With the discovery of this novel function for NR, the biological significance of NR as a NO emitter and the induction of NR in response to pathogen attack or other stimuli known to induce NO production are drawing more and more attention in recent years.

1.3 Regulation of NR

Assimilatory nitrate reductase of higher plants is tightly regulated in a very complex manner. Some of the regulatory patterns are summarized in Figure 1.2. Nitrate

reductase is regulated at both the transcriptional and the post-translational level. The regulation of NR expression by nitrate, light and carbohydrates, the mechanism of its post-translational modulation and the role of its N-terminal domain in that regulation are described in the following sections.

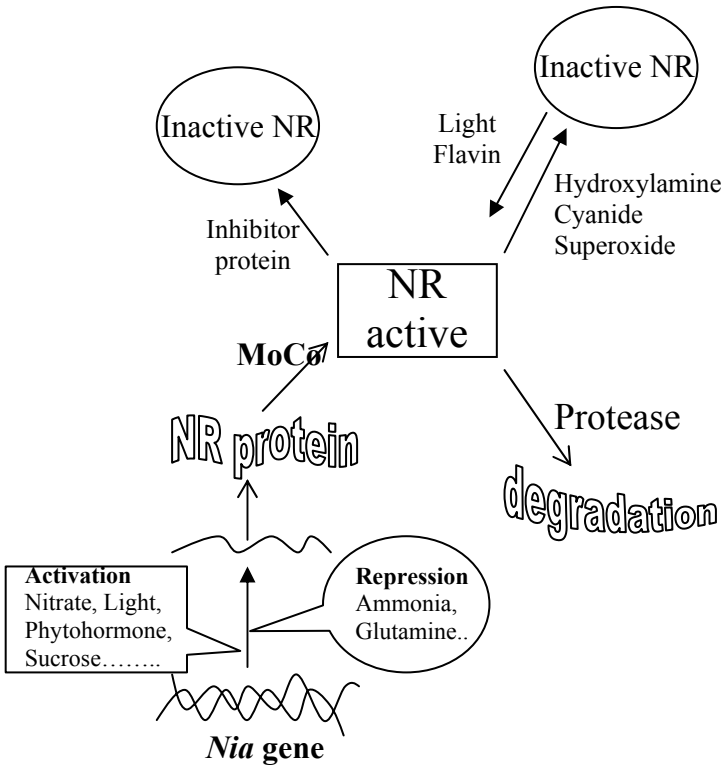

Figure 1.2 Schematic summary of the regulation of NR. The various signaling pathways include positive and negative effectors for gene expression and for post-transcriptional regulation, NR protein synthesis and degradation and Molybdenum cofactor (Moco) incorporation (Solomonson, 1990; modified).

1.3.1 Transcriptional regulation of NR

1.3.1.1 By Nitrate

As a signal, nitrate reprograms nitrogen and carbon metabolism and the expression of a selected group of genes (for reviews, see Crawford, 1995; Stitt, 1999; Tischner, 2000). It has been known for decades that nitrate uptake and nitrate reductase activity increase after adding nitrate. In the absence of nitrate, NR mRNA was not detected in leaves and only low levels were present in barley roots, but could be detected in roots and leaves within 40 min after supplying nitrate to roots (Melzer et al., 1989). Very recently, microarray and RNA gel blot analyses were performed to identify Arabidopsis genes that responded to nitrate. The overall finding was that genes involved directly or indirectly in nitrate and nitrite reduction were the most highly

induced by nitrate. These results are not difficult to understand, because nitrite is toxic to plants and is usually not allowed to accumulate (Wang et al., 2000).

Pretreatment of seedlings with cycloheximide to block cytosolic protein synthesis did not prevent nitrate induction of *Nia* mRNA in maize leaves (Gowri et al., 1992). In the case of Arabidopsis, nitrate increased the transcription of both *Nia1* and *Nia2* genes. The 5' flanking regions of *Nia1* deleted to -238 (NP1) and *Nia2* deleted to -330 (NP2) were demonstrated to be sufficient to confer reporter gene expression comparable to that of the entire regions prior to deletion (Lin et al., 1994). A further identification of the positive nitrate-response elements within NP1 and NP2 by performing a linker-scanning (LS) analysis has been reported (Hwang et al., 1997). Additionally, a cis-acting element which is necessary for nitrate-dependent transcription has been found in the promoter of NR gene from birch (Strater and Hachtel, 2000).

1.3.1.2 By Light and carbohydrates

Light is a major factor enhancing expression of NR genes as well as of many carbon fixation genes at different levels. In many species, *Nia* mRNA was rapidly increased when etiolated plants or dark-adapted green plants were transferred to light.

In etiolated squash cotyledons, a red light pulse induced NR mRNA and a far-red light pulse reversed the induction (Rajasekhar et al., 1988). Blue light also induced NR mRNA accumulation in etiolated barley seedlings (Melzer et al., 1989). These limited findings suggest that light, acting via the photoreceptor phytochrome and perhaps the blue-light receptor plays a direct role in NR gene regulation in etiolated plants. However, Arabidopsis plants those were in continuous darkness for 16 days and were supplemented with sucrose, accumulated *Nia1* mRNA to the same extent as those grown in light conditions. This is an indication that sucrose alone is sufficient to induce the full expression of the nitrate reductase gene in etiolated Arabidopsis plants. A subsequent exposure of the plants to light lead to no increase in *Nia1* mRNA after 12 hr of light treatment, some increase was observed after 24 hr (Cheng et al., 1992).

In green plants, the effect of light is quite different from that on the etiolated seedlings. Only white light, but not red or blue light, stimulated NR mRNA accumulation in barley seedlings (Melzer et al., 1989). Using dark-adapted green plants, the Arabidopsis *Nia1* gene was induced by white light. Like in the case of etiolated plants, sucrose could replace light in eliciting an increase of *Nia1* mRNA accumulation in

dark-adapted green Arabidopsis plants (Cheng et al., 1992). Besides sucrose, glucose or fructose also induced NR mRNA as well as NR protein and activity in detached leaves of dark-adapted, sugar-depleted tobacco plants (Vincentz et al., 1993).

Vincentz and Caboche (1991) demonstrated that CaMV 35S-NR transgenic tobacco plants accumulated high levels of the transcript that was not affected by dark treatment, indicating that light indeed plays a role in transcriptional regulation of NR gene. Furthermore, a 2.7-kb region of the 5' flanking sequence of the Arabidopsis *Nia1* promoter could confer the light and sucrose response (Cheng et al., 1992), and the tobacco *Nia1* promoter expressed the reporter gene in a sugar-inducible way (Vincentz et al., 1993). More recently, the light-responsive region of NR promoter from birch was identified (Strater and Hachtel, 2000).

On the other hand, during a 24 hr light-dark regime, it has been reported that both tomato and tobacco NR mRNA increased drastically before the end of the dark period and reached a maximum at the beginning of the day. At the end of the day, the level of NR mRNA was then markedly decreased. No obvious correlation was detected between levels of NR mRNA and NR activity (Galangau et al., 1988). A similar phenomenon was also found in maize leaves in which NR mRNA levels were low at the end of the dark period, peaked within 2 h in the light and decreased thereafter, whereas NR activity generally remained high (Huber et al., 1994). Apparently, the positive feed-forward induction of NR genes by sugars, and the negative feedback by nitrogen compound(s) from nitrate assimilation results in the diurnal oscillation of NR mRNA.

1.3.2 Post-transcriptional regulation of NR

1.3.2.1 By nitrate

In addition to the transcriptional control of NR genes by nitrate, NR activity is also affected by nitrogen availability at the post-transcriptional level. One example from earlier studies was that in tomato plants after 8d of growth in the absence of nitrate, NR activity and protein decreased to lesser than 10% of the initial values, while *Nia* mRNA remained at a level close to normal (Galangau et al., 1988). More recently, tobacco suspension cells were used to determine the response of NR activity to exogenous nitrate levels. In this case, a long-distance transport of nitrate from roots to shoots is not required. The NR activity was found to increase as cell nitrate increased. The increase of NR activity appeared related, but not directly proportional, to the

intracellular level of nitrate (Zhang and MacKown, 1993). On the other hand, when nitrate supply to barley plants stopped and the internal nitrate concentrations became extremely low, NR protein and activity decreased very drastically during the day (Man et al., 1999).

1.3.2.2 By light and carbohydrates

Kaiser and Förster (1989) demonstrated that nitrate reductase activity in spinach leaves was rapidly decreased within minutes when rates of photosynthesis were decreased, e.g. by closure of stomata, or after lowering the external CO_2 concentration. . NR activity decreased rapidly to about 15% of the control with a half-time of only 2 min after darkening of spinach leaves (Riens and Heldt, 1992). This rapid response to darkness shifting might help plants to avoid a build-up of nitrite, which is toxic to the cells. The inactivation of NR in response to CO_2-deficiency indicates that photosynthetic fixation of CO_2 appeared necessary for maximal NR activity (Klepper et al., 1971; Kaiser and Brendel-Behnisch, 1991). Additionally, CO_2 enrichment markedly elevated nitrate reductase activity in young, but not old, tobacco plants, presumably because growth rates were much greater in the younger plants and they needed the extra nitrogen provided by this phenomenon to maintain their heightened level of development (Geiger et al., 1998). The demonstration that light is an effective regulator of NR at the post-translational level by reversible protein phosphorylation (Kaiser et al., 1992; Huber et al., 1994), has been a major breakthrough in our knowledge on NR regulation. Thus, more details on this aspect are given below.

1.3.3 Mechanism of the post-translational regulation of NR in higher plants

A remarkable discovery in recent years has been the demonstration that *in vivo* NR activity is rapidly modulated by reversible protein phosphorylation and 14-3-3-binding in the presence of divalent cations (Figure 1.2; for reviews, see Kaiser and Huber, 2001; MacKintoch and Meek, 2001; Kaiser et al., 2002).

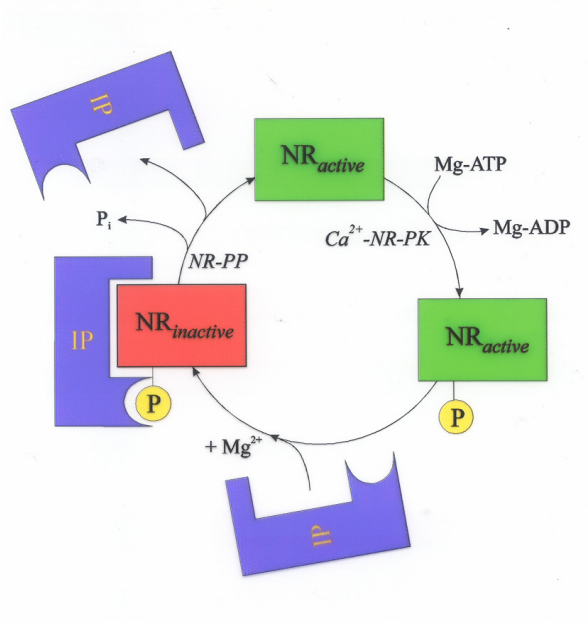

Figure 1.3 Post-translational regulation of NR. Explanations in the text.

As already mentioned, NR activity in leaf extracts was rapidly reduced when plants were transferred to darkness or to low CO_2 and NR activity was restored when the light was turned on or when plants were brought back to normal CO_2 levels (Kaiser and Brendle-Behnish, 1991). Subsequently, Kaiser and Spill (1991) found out that NR was probably regulated by reversible protein phosphorylation. They further found that phosphorylation of NR *per se* was not sufficient for its inactivation, and two proteins were partially purified that appeared involved in the ATP-dependent inactivation process (Spill and Kaiser, 1994). In 1995, a protein termed "inhibitor protein (IP)" or "NR inhibitor protein (NIP)" was found, eluting with about 70 kDa from a sizing column (Bachmann et al., 1995; Glaab and Kaiser, 1995; MacKintosh et al., 1995). The "NR inhibitor protein" inactivated only phospho-NR, and this inactivation required divalent cations. After immunopurification and sequencing of a tryptic peptide, IP was identified for the first time as a member of the eukaryotic 14-3-3 protein family (Bachmann et al., 1996a). Meanwhile, many studies highlight how phosphorylation inhibits NR activity, by identifying the regulatory phosphorylation site in the NR molecule and by determining the association between 14-3-3s and spinach phospho-NR in an isoform specific manner (Douglas et al., 1995; Bachmann et al., 1996b and 1996c; Moorhead et al., 1996; Su et al., 1996). Hence, a two-step regulation model of spinach NR was proposed (compare figure 1.3). According to this

model, spinach NR is first phosphorylated on Ser-543, which is conserved among higher plant NRs, by a Ca^{2+}-dependent NR-kinase (Douglas et al., 1997 and 1998) and then becomes inactivated upon binding of 14-3-3s in the presence of divalent cations. Therefore, in the presence of mM concentrations of free Mg^{2+}, the remaining enzymatic activity represents that of the native free NR (NR_{act}). In contrast, with excess EDTA, no 14-3-3-binding occurred and the measured activity therefore reflects the total amount of NR (NR_{max}). Originally it appeared that 14-3-3s bind directly to phosphoserine and divalent cations promote this binding (Athwal et al., 2000). However, it is not yet certain, whether divalent cations are required for 14-3-3 binding to phospho-NR, or whether they are needed for switching the complex from an active into an inactive form or both (Weiner and Kaiser, 2000).

1.3.4 The role of the N-terminal domain in NR regulation

Most of the NR protein sequence is well conserved among higher plants and other organisms, apart from the N-terminal region, which varies both in sequence and length among species. Therefore, to elucidate the potential role of the N-terminal domain in higher plants may contribute to unravel the complex networks of NR regulation.

It has been shown that a tobacco NR with an internal deletion of 56 amino acids in the N-terminal domain (termed ΔNR) was no longer regulated by light-dark transition. Placing wild-type and transgenic plants C1 (transformed with CaMV 35S-NR construct) or *del* (transformed with CaMV 35S-ΔNR construct) in the dark for 30 min revealed that *in vivo* inactivation by darkness occurred in wild-type and C1, but was completely absent in ΔNR plants. Moreover, *in vitro* inactivation by MgATP was also abolished in ΔNR plant extracts (Nussaume et al., 1995). However, ΔNR appeared to be phosphorylated in the dark and endogenous 14-3-3 proteins could copurify with both NR and ΔNR (Lillo et al., 1997; Provan et al., 2000), indicating that they bound to both forms.

Loss of the first 45 amino acids by proteolysis during purification of spinach NR resulted in an enzyme that could no longer be fully inhibited by 14-3-3-binding, although the truncated enzyme could be phosphorylated by NR kinase on Ser-543 at the same rate as intact NR (Douglas et al., 1995).

The 56 amino acid region within the N-terminal domain of NR contains an "acidic stretch" conserved only in higher plants. Removal of the acidic stretch led to an active

enzyme which was more thermosensitive than the wild-type NR, but it was relatively insensitive to the inactivation by phosphorylation in the dark. The acidic stretch seemed to be required but not sufficient for the inactivation of NR (Pigaglio et al., 1999). In addition, the deletion of 56 amino acids in the N-terminal domain showed that NADH to CcR activity (needs active FAD and heme-binding domains, compare Figure 1.1) was similar in C1 and *del* plants, but the terminal activity (associates with the MoCo-binding domain) was inactivated, indicating that the N-terminal region may be important for stabilizing the Moco-binding domain (Provan et al., 2000).

Taken together, these data suggest that the N-terminal region of NR is in some way involved in and is required for the inactivation of the enzyme by phosphorylation. However, the exact role of this region is still under debate.

1.4 An exceptional example from Ricinus

Ricinus communis, or Castor Bean, is not a true bean, but a member of the spurge family or Euphorbiaceae. It is a very fast growing plant, it habitats ranging from cool temperate through tropical desert to wet forest life zones. Ricinus is reported to tolerate annual temperatures of 7.0 to 27.8°C and a wide range of soil pH from 4.5 to 8.3 (Duke, 1983).

In a previous study, NR from leaves and roots of hydroponically grown Ricinus behaved differently from all other NR studied so far (Kandlbinder et al., 2000). So far, NRs from different taxa of higher plants followed the light-dark transition or the diurnal fluctuation pattern, in which both NR_{act} and NR_{max} were low during the night and increased during the first half of the day, with a significant decrease during the second half. In Ricinus, however, NR_{max} (indicating total active NR protein) underwent a similar pattern as, for example, spinach NR, whereas NR_{act} was always extremely low with only little difference in light and dark. Therefore, the resulting activation state ($NR_{act} \times 100/NR_{max}$) of Ricinus NR was always very low, usually less than 10% of NR_{max} throughout the day.

Secondly, Ricinus NR had a different pH-profile in comparison with spinach NR. NR_{act} in extracts from Ricinus was strongly increased by pH values below 7, and was extremely low at pH ≥ 7.3. This effect was actually caused by pH-dependent changes in the Mg^{2+}-sensitivity of Ricinus NR. At pH 7.6 and pH 6.8, 50% inhibition of NR in spinach leaf extracts occurred at 3 mM and 1 mM Mg^{2+}, respectively. With Ricinus

leaf extracts, 50% inhibition was obtained at less than 0.2 mM Mg^{2+} at pH 7.6, but 5 mM Mg^{2+} was required at pH 6.8.

Moreover, NR from spinach and other plants can be rapidly inactivated *in vitro* in the presence of MgATP. As described above, this inhibition requires protein kinase and 14-3-3s. However, preincubation of Ricinus NR with MgATP gave very little additional inactivation. Removal of 14-3-3s by partial purification of NR, which activates NR from spinach, did not activate Ricinus NR. When pH values above 7, Ricinus NR appeared very Mg^{2+}-sensitive and this high Mg^{2+}-sensitivity does not require 14-3-3's binding as in other plant NRs. On the other hand, peptide antisera against the sequence around serine 543 of the spinach-NR cross-reacted with Ricinus NR indicating that Ricinus NR possesses the same potential 14-3-3 binding motif as spinach NR. Therefore, the molecular basis and the physiological relevance for the deviating properties of the Ricinus enzyme are not yet understood.

1.5 Objective of this thesis

The basic objective of this thesis was to elucidate the reasons behind the unusual regulatory properties of nitrate reductase from *Ricinus communis* L. both with respect to molecular and physiological aspects

For that purpose, the first aim of this study was to clone the NR gene from Ricinus and eventually use a heterologous expression system to study the molecular properties of Ricinus NR, and to investigate the following problems:

(1) Does the primary sequence of Ricinus NR differ from other NRs?

Once the Ricinus NR gene was obtained, its deduced amino acid sequence could be used for sequence alignments to predict whether Ricinus NR would contain any unusual motifs or unique residues. For example, is the N-terminus of Ricinus NR sufficiently different from other NRs to provide its unresponsiveness to MgATP (as in the N-terminally deleted tobacco mutants where the ATP inactivation is abolished; Nussaume et al., 1995)? Furthermore, does a potential 14-3-3 binding motif exist in the Ricinus NR sequence, as already indicated by immunological studies?

(2) Heterologous expression of Ricinus NR in *Pichia pastoris*

Transformation of the Ricinus NR gene into a heterologous expression system would provide an easy way to purify this enzyme for functional and regulatory studies. In addition, a heterologous expression system would be also advantageous for mutagenesis experiments.

(3) Characterization of recombinant Ricinus NR

Biologically similar recombinant NR's (e.g. Arabidopsis) expressed in *Pichia* could be used for a comparison with Ricinus NR from the same expression system. This would provide insight whether the different features of Ricinus NR are based on the NR protein itself or on some other factors.

2 Results

2.1 Molecular cloning of NR gene from Ricinus

2.1.1 The primary Ricinus NR gene sequence obtained by RT-PCR

A partial Ricinus NR cDNA was obtained by RT-PCR (reverse transcription-polymerase chain reaction). The main limitation of this technique is the need for precise sequence information from the target gene for the design of specific and efficient PCR primer. Therefore, based on sequence comparison we choose two regions where the amino acid sequences are entirely identical in 10 different NR-sequences. The deduced protein sequences of these two regions are "IGGRMVKW" and "GMMNNCWF" which locate at 316 to 323 and 484 to 491 in spinach, respectively. The *Ricinus communis'* codon usage was subsequently used to backtranslate these amino acid sequences into nucleotide sequences and for the design of sense-primers. RT-PCR with RcNR-S1 (backtranslate from IGGRMVKW) and an appropriate antisense-primer from Arabidopsis *Nia1* sequence yielded a cDNA-fragment of the expected size of ca. 440 bp (Figure 2.1). Subsequently, this PCR product was cloned and sequenced and compared to database entries. The sequence shared high homology with NR known from many higher plants in the GenBank.

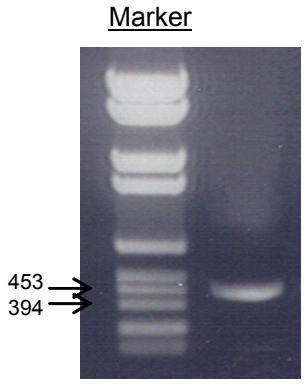

Figure 2.1 A cDNA fragment of Ricinus NR gene was amplified by RT-PCR. Total RNA was used as the template for reverse transcription. Combination of the specific sense-primer for Ricinus NR gene and an antisense-primer from Arabidopsis *Nia1* gene revealed a ca. 440 bp cDNA fragment. The first lane is a DNA-marker with two indicated fragments, 453 and 394 bp.

2.1.2 The Full-length Ricinus NR gene

Since the primary sequence of NR from Ricinus is known, we used so-called 5'-RACE and 3'-RACE (Rapid Amplification of cDNA Ends) to obtain the full-length Ricinus NR cDNA. 25mer gene specific primers for 5'-RACE (RcNR-AS-RACE) and 3'-RACE (RcNR-S-RACE) PCR reaction were chosen from the partial Ricinus cDNA sequence mentioned above. Total RNA from Ricinus leaves was used as starting material for the amplification of full-length cDNA (Figure 2.2).

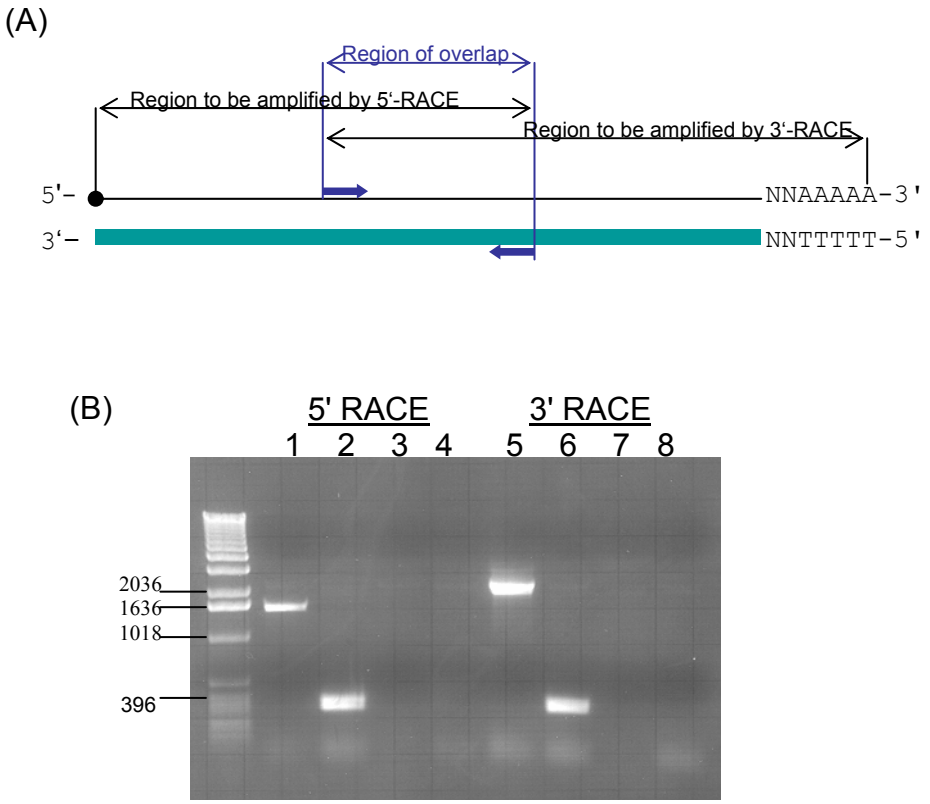

Figure 2.2 Amplification of Ricinus NR cDNA fragments by 5'-RACE and 3'-RACE PCR reaction. (A) Schematic representation of the relationship of gene specific primers to the cDNA template. (B) Analysis of PCR products from RACE. Lane 1 and lane 5 represent the Ricinus NR cDNA fragments amplified through 5'- and 3'-directions. Lane 2 and 6 show the overlapping region amplified by the gene specific primers. Lane 3, 4, 7 and 8 are the negative control which used only one of the primer pairs used in 5'- and 3'-RACE. DNA-markers with indicated sizes are shown on the left side.

Combination of the cDNA fragments amplified by 5'-RACE and 3'-RACE revealed a length of 3021 nucleotides. An open reading frame of 914 amino acids was encoded, starting with the sequence MAASV that is conserved throughout all higher plant

nitrate reductases (Miyazaki et al., 1991) except NRs from maize (*Zea mays*, MSTCV), Arabidopsis NR1 (MATSV) and *Brassica napus* (MATSV).

2.1.3 Analysis of the deduced amino acids sequence

Searching for the ORF of the Ricinus NR sequence against the Conserved Domain Database with the RPS-BLAST program revealed that Ricinus NR can be divided into distinct functional domains that bind a molybdenum-pterin cofactor (Moco), heme-Fe or FAD (flavin adenine dinucleotide) as in other higher plants. In Ricinus NR, the three prosthetic domains Molybdenum-MPT, heme-Fe and FAD are located at amino acid positions 92 to 321, 536 to 612 and 661 to 768 and are composed of 230, 77 and 108 amino acids, respectively (Figure 2.3).

```
Ricinus-NR : MAASVDNRQFH.LEPTLNGVVRPFKSGPTHRSDSPIRNGFNFTNQDFTR. :  48
Arabidopsi : ----------YAR---G------SY-PPVPG-----KAHQNQT---TVFL. :  49
Spinach-NR : ------.--Y-.PA-.MS----TPF-N.H------V---YT-S-PPSSNG :  46

Ricinus-NR : ............SSSNKKPTPIMDDDFSSDDDEAD.......YGDLIRK :  78
Arabidopsi : ..............KPAKVHDD-E-V--E-ENETHNSNAVY-KEM--- :  83
Spinach-NR : VVKPGEKIKLVDNNSNSNNGSNNNNNRYD--SE-D-DENEMNVWNEM-K- :  96

Ricinus-NR : GNSELQPSILDPRDEGTADNWVERHPSMIRLTGKHPFNSEAPLTQLMHHG : 128
Arabidopsi : S-A--E--V------Y---S-I--N---V--------------NR----- : 133
Spinach-NR : -----E--SV-S-------Q-I--N---------------P---R----- : 146

Ricinus-NR : FITPVPLHYVRNHGPVPKASWKDWTVEICGLVKKPTRFTMDQLVNDFPSR : 178
Arabidopsi : --------------H----Q-AE----VT-F--R-MK-------SE-AY- : 183
Spinach-NR : -L---------------N-K-E-----VT----R-I-----------Q-- : 196

Ricinus-NR : ELPVTLVCAGNRRKEQNMVKQTIGFNWGAAGVSNSVWRGVPLHFVLKRCG : 228
Arabidopsi : -FAA----------------KSK-----S----T--------CD--R--- : 233
Spinach-NR : -F----------------T--S------S-A--T--------RD------ : 246

Ricinus-NR : IYSRKKGALNVCFEGAEDLPGGG...GSKYGTSIKKEYAMVPSRDIILAY : 275
Arabidopsi : -F---G---------S------AGTA--------------D--------- : 283
Spinach-NR : VM-SL------------------...-------V-R-F-D--A------- : 293

Ricinus-NR : MQNGELLAADHGFPVRMIIPGFIGGRMVKWLKRIIVTTKESDNYYHYKDN : 325
Arabidopsi : -----Y-TP-------I--------------------------F--F--- : 333
Spinach-NR : -----K-SP---Y------------------------T----------- : 343

Ricinus-NR : RVLPSHVDAELANAEAWWYKPEYIINELNINSVITTPSHEEVLPINSWTT : 375
Arabidopsi : -----L------DE-G---------------------C---I----AF-- : 383
Spinach-NR : -------------S------Q--------V-----S-C---I----A--- : 393

Ricinus-NR : QRPYTLKGYAYSGGGKKVTRVEVTMDGGDTWQVCSLDHPEKPNKYGKYWC : 425
Arabidopsi : ------------------------V---E--N--A---Q--------F-- : 433
Spinach-NR : -----MR--------R---------------DI-E---Q-RGS----F-- : 443
```

```
Ricinus-NR : WCFWSLEVEVLDLLGAKEIAVRAWDETLNTQPEKLNWNVMGMVNNCWFRI : 475
Arabidopsi : --------------S-------------------MI--L---M------V : 483
Spinach-NR : -------------------G------S--------I------M------V : 493

Ricinus-NR : KTNVCKRHKGEIGIVFEHPTVPANQSGGWMAKERHLEISSENHPILKKSV : 525
Arabidopsi : ------P-------------L-G-E------------K-ADAP-S----- : 533
Spinach-NR : ------P-------------Q-G-K------R-------.DSG-T--RTA : 542

             *
Ricinus-NR : STPFMNTSSKTVSMAEVKKHNSADSCWIIVHGHVYDCTRFLKDHPGGTDS : 575
Arabidopsi : -------TA-MY--S-----------------I-------M-----S-- : 583
Spinach-NR : -------T--MY-S-------T---A--V---N--NA----------S-- : 592

Ricinus-NR : ILINAGTDCTDEFDAIHSDKAKIMLEDYRIGELVDSTAYTTDSNASSPNN : 625
Arabidopsi : ----------E--E--------K----------IT.-G-SS--..----- : 630
Spinach-NR : ----------E-----------RL---F-----IS.-G--S--..---G- : 639

Ricinus-NR : SVHGASNMS.QTPLAPIKEITPTPARNVALV.PREKIACKLVKKESLSHD : 673
Arabidopsi : ----S-AVF..SL----G-A--..V--L---N--A-VPVQ--E-T-I--- : 676
Spinach-NR : ----G-VY-GLAG----T-AV-..L-----N.--V--P---IE-V----- : 686

Ricinus-NR : VRLFRFALPSDDQVLGLPVGKHIFLCATIDEKLCMRAYTPTSTIDVVGYF : 723
Arabidopsi : --K------VE-M----------------ND---L-----S--V------ : 726
Spinach-NR : --R---G---E----------------NV-D---------S--------- : 736

Ricinus-NR : DLVIKVYFKGVHPKFPNGGLMSQHLDSLQLGSVIDVKGPLGHIEYAGRGN : 773
Arabidopsi : E--V-I--G----R---------Y----PI--TLEI------V--L-K-S : 776
Spinach-NR : ---V-----D---R-----V--------S---IV----------L-K-- : 786

Ricinus-NR : FMVHGKPKFAKKLTMLAGGTGITPIYQVIQAILKDPEDDTEMYVVYANRT : 823
Arabidopsi : -T--------D--A----------V--I----------E-----I----- : 826
Spinach-NR : -T-----------A-IS----------M---------K---H------- : 836

Ricinus-NR : EDDILLREELDSWAKEHHERLKVWYVVQESIKEGWQYSVGFITENILREH : 873
Arabidopsi : -E---------G--EQYPD--------ES.A----A--T---S-A-M--- : 875
Spinach-NR : -E---------K--D-FRD-V------EK.AE---K-DT---S-K---D- : 885

Ricinus-NR : VPEGSD.DTLALACGPPPMIQFAVQPNLEKMNYDIKNSLLVF.. : 914
Arabidopsi : I-D-L-GSA--M-------------------Q-N--EDF-I-.. : 917
Spinach-NR : --AVG-.-V---T---------------D--GF---EQ--I-.. : 926
```

Figure 2.3 Deduced amino acid sequences alignments. Amino acid sequences of Arabidopsis NR2 and spinach NR were compared to Ricinus NR (GenBank accession number AF314093). Like other plant NRs, Ricinus NR consists of three prosthetic domains which are marked by a shadow. The Mo-MPT domain with the nitrate-reducing site spans from 92 to 321, the cytochrome b domain from 536 to 612 and the FAD domain from 661 to 768. Identical sequences are indicated by a dash, and gaps introduced for alignment purposes by a dot. Amino acids in a block represent the 14-3-3 binding motif, where the phosphorylated serine residue is marked by an asterisk. A sequence involved in the character for a NADH:NR is underlined.

The deduced amino acid sequence showed that Ricinus NR mRNA encodes a protein of 102.8 kDa with a high degree of similarity to NRs from other higher plants. The

highest similarity was found to the NR amino acid sequence from winter squash (78% identity, 87% similarity), petunia (78% identity, 86% similarity), tomato (77% identity, 87% similarity) and tobacco (78% identity, 87% similarity). Moreover, Ricinus NR shared 75% identity (85% similarity) with Arabidopsis NR2 or spinach NR and 73% identity (84% similarity) with Arabidopsis NR1.

In contrast to the larger part of the sequence, the N-terminal regions of the NR sequences varied considerably (see Appendix 6.5). The molybdenum-MPT domain of Ricinus NR for example contains a cysteine residue (Cys-186) which is present in all eukaryotic NRs and is a part of a highly conserved region ^{183}TLVCAGNRRKEQNM^{196} (Figure 2.3). This key residue has been proposed to provide a ligand to molybdenum in the Moco binding region and is essential for NR activity (Barber and Neame, 1990; Solomonson and Barber, 1990; Garrett and Rajagopalan, 1994; Garde et al., 1995; Su et al., 1997).

In the hinge region between molybdenum-MPT and heme-Fe domains, Ricinus NR possesses a conserved phosphorylation serine residue (Ser-526), which is within the 14-3-3s binding motif ^{523}KSVS*TP^{528} (Figure 2.3). This confirmed the result from our previous immunodecoration experiment where peptide antisera against the sequence around serine 543 of the spinach-NR cross-reacted with Ricinus NR (Kandlbinder et al., 2000). Moreover, within the FAD domain Ricinus NR possesses the motif ^{886}CGPPP^{890}, characteristic for NADH-specific NRs (Schöndorf and Hachtel, 1995). In NAD(P)H NR-forms such as those of barley and birch only two proline residues are present (CGPPA). It has been demonstrated that substitution of a proline by alanine in the birch NAD(P)H-NR greatly increased preference for NADH. In the case of Ricinus NR, we have tested whether the recombinant enzyme could utilize NADPH as the reductant, but the recombinant protein had no NADPH:NR activity (not shown).

2.2 Heterologous expression of RcNR in *Pichia pastoris*

2.2.1 Construction

The full-length cDNA of Ricinus NR (RcNR) was cloned into the pPICZA *Pichia pastoris* expression vector using *Sac* II and *Apa* I restriction enzyme sites. The plasmid was further transformed into *E. coli* for replication. The first evidence for the successful construction of pPICZA-RcNR clone was using gene and vector specific primers to check the construct by PCR (Figure 2.4).

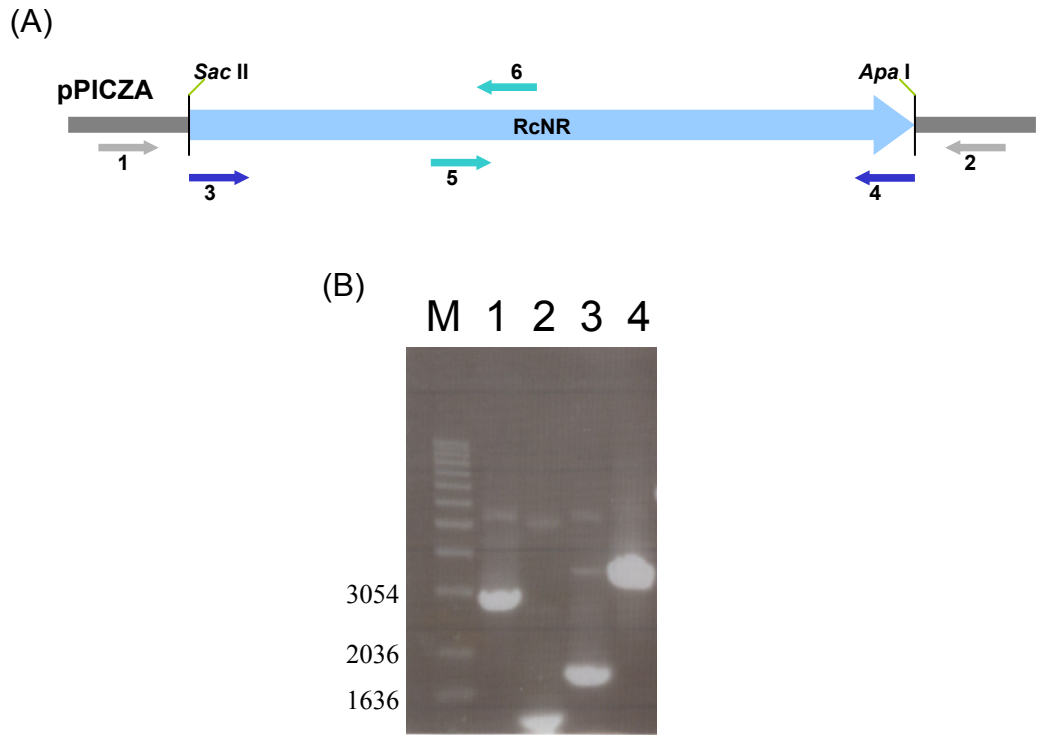

Figure 2.4 Determination of the construct pPICZA-RcNR by PCR. Primers specific for the Ricinus NR gene and the vector pPICZA were chosen to perform the PCR reaction using the plasmid pPICZA-RcNR as the template. (A) Schematic representation of the construct pPICZA-RcNR and the positions of the primers. (B) Analysis of the PCR products using 1% agarose gel. M: DNA-marker. More details in the text.

Primers 1 and 2 (5' and 3' *AOX1*) were vector specific which would amplify a DNA fragment of 2977 bp (see lane 4, Figure 2.4 B). Primers 3 to 6 were specific for the Ricinus NR gene. Combination of primers 3 and 4 (RcNR-SF-2 and RcNR-ASF) revealed a full-length RcNR DNA fragment of 2764 bp (lane 1, Figure 2.4 B). Using internal gene specific primer 5 (RcNR-S-RACE) together with primer 4 amplified a DNA fragment of 1736 bp (lane 3, Figure 2.4 B). Another combination of primer 3 and 6 (RcNR-AS-RACE) amplified a DNA fragment of 1344 bp (lane2, Figure 2.5 B). These precise DNA fragments amplified by gene and vector specific primers confirmed that the construct was successfully created.

The second evidence was the digestion pattern of the plasmid by *Nco* I restriction enzyme. Ricinus NR cDNA contains 3 *Nco* I restriction enzyme sites as well as one in pPICZA vector. Therefore, after *Nco* I digestion it would reveal 4 DNA fragments if the construct was correct (Figure 2.5). The predicted size of 4 DNA fragments is 2928,

1968, 874 and 291 bp (which is in total 6061 bp), respectively. Analysis of the digestion mixture revealed that 4 DNA fragments were precisely produced by *Nco* I digestion.

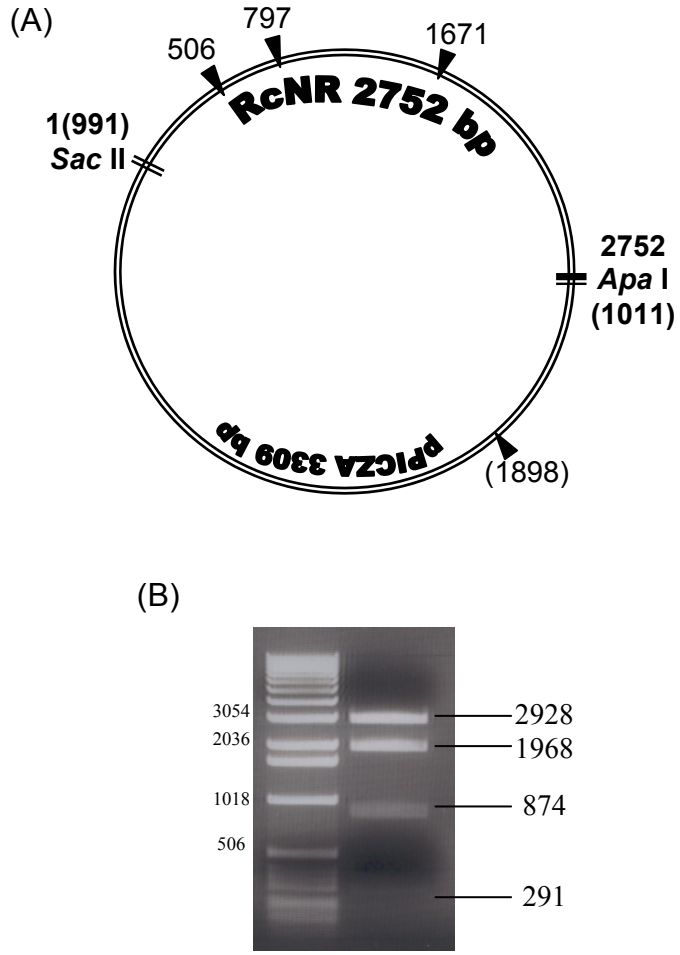

Figure 2.5 *Nco* I restriction enzyme digestion of the construct pPICZA-RcNR. Panel A illustrated the restriction enzyme sites of *Nco* I in the pPICZA-RcNR construct. Triangles labeled with numbers represent *Nco* I enzyme sites. Numbers with or without brackets indicate the positions corresponding to the pPICZA and RcNR sequence, respectively. Panel B showed the verification of DNA fragments after digestion of *Nco* I in 1% agarose gel. Sizes of the DNA-marker and the PCR fragments are indicated.

In addition to the evidence mentioned above, the construct was also sequenced by using appropriate sequencing primers. The partial sequence of the construct obtained from the sequencing revealed that the cloning process was successful. It is noteworthy that in the construct a yeast consensus sequence was added to the RcNR sequence

right before the initiation ATG. This sequence is necessary for the yeast to initiate the translation and also appeared in the partial sequence (not shown).

2.2.2 Transformation

Having confirmed the correct construct pPICZA-RcNR, it was then linearized with *Sac* I restriction enzyme, transformed in *P. pastoris* to be integrated into the *P. pastoris* genome. At this stage, a direct PCR screening was used to identify whether the transformation was accomplished. *Pichia* transformants were tested for insertion of the Ricinus NR gene by using 5'- and 3'-*AOX1* sequencing primers located at the vector pPICZA, as well as the internal gene specific primers (Figure 2.6).

The unique *Sac* I restriction site would permit linearization of the construct at the *AOX1* locus for efficient integration into the *Pichia* genome. After the digestion of *Sac* I restriction enzyme, the construct was linearized and revealed a total length of 6061 bp (Figure 2.6A). This linearized plasmid was then used for transformation by using chemically produced competent *Pichia* cells and yielded 12 colonies. It is worth to mention that this alternative to electroporation is rapid and convenient but the transformation efficiency is low (3 μg plasmid DNA yields about 50 colonies). Genomic DNA of *Pichia* transformants, in the case of direst PCR screening, was used as the template and tested by PCR. The internal gene specific primers and 5'- and 3'-*AOX1* sequencing primers should amplify DNA fragments of 1344 and 2977 bp, respectively (Figure 2.6 B and C). However, using 5'- and 3'-*AOX1* sequencing primers, an additional DNA fragment of about 2 kb was also amplified. No homologous sequence to 5'- and 3'-*AOX1* sequencing primers was found in the Ricinus NR gene.

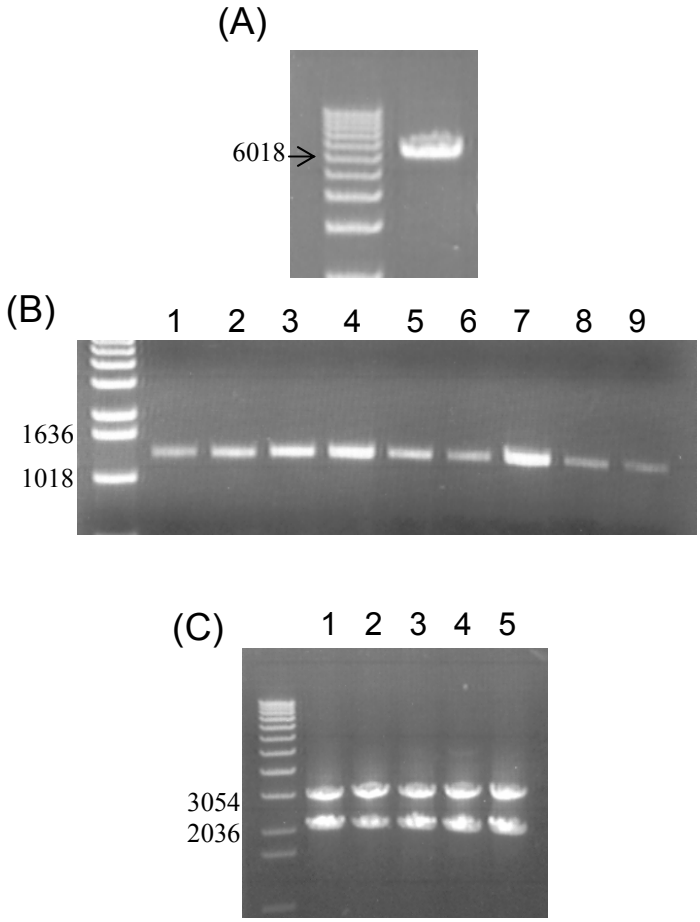

Figure 2.6 Linearization of the construct pPICZA-RcNR and direct PCR screening of *Pichia* clones. *Pichia* cells were lysed by a combined enzyme, freezing and heating treatment. The genomic DNA was used directly as a PCR template. (A) Linearized construct with *Sac* I restriction enzyme before transformation. (B) After transformation, *Pichia* transformants 1 to 9 were tested for the insertion of RcNR by using internal gene specific primers. (C) Selected *Pichia* clones 1 to 5 were further tested, using 5'- and 3'-*AOX1* sequencing primers.

2.2.3 Expression

The *P. pastoris* alcohol oxidase promoter (*AOX1*), which is inducible by methanol and produces alcohol oxidase up to 30% of total soluble protein, was used to drive the expression of NR cDNA. During a 72 hr induction period, transformants were collected for checking protein expression levels by SDS polyacrylamide gel electrophoresis (SDS-PAGE). However, proteins produced were not detected in a Coomassie-stained SDS-PAGE. Indeed, we always observed only an extremely weak Ricinus NR protein signal in the predicted molecular weight (MW) region, but very strong signals appeared in the lower MW region on the SDS-PAGE. Moreover, using

different peptide antisera to perform western blots revealed no successful results so far. One of the antisera raised against the maize NR peptide (PAESDNYYHFKDNRVLPSC) could recognize purified maize NR but failed to cross-react with recombinant Ricinus NR and Arabidopsis NR2, which was expressed in the same system. We also tried the other antisera raised against the spinach NR peptide (CGPTLKRTADTPFMNTTS), however, no signals of the predicted size for both, recombinant Ricinus and Arabidopsis NRs were detected.

In order to test whether *P. pastoris* transformed with the NR expression plasmid pPICZA-RcNR could produce a functional enzyme, NR activity was measured in yeast cells (*in vivo*). *Pichia* transformants were induced with methanol within a 72 hr induction period. *Pichia* transformed with plasmid pPICZA-RcNR showed NR activity, whereas *P. pastoris* containing only a control expression plasmid (pPICZA) had no such activity throughout the induction period (Figure 2.7). The *in vivo* activity was compared with the recombinant Arabidopsis NR2 from the same system but using a different expression vector.

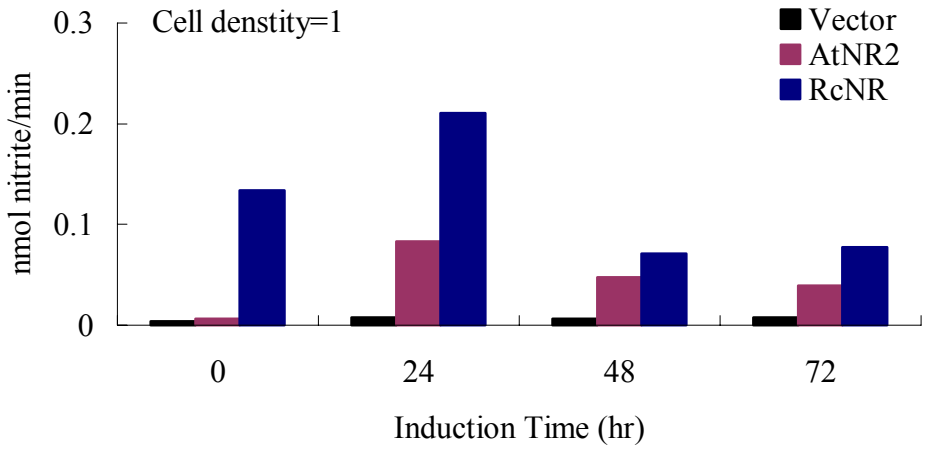

Figure 2.7 NR activity in yeast cells (*in vivo*). *Pichia* transformed with NRs from either Arabidopsis (pHILD2-AtNR2) or Ricinus (pPICZA-RcNR) and the expression vector (pPICZA) were tested for the NR activity *in vivo*. After incubation of the cell suspension (OD_{600}=1) with substrate (50 mM KNO_3), the nitrite content in the medium was visualised with the Griess Reagent.

Before transferring *Pichia* transformed with the plasmid pPICZA-RcNR to the induction medium to induce expression, the Ricinus NR gene was already expressed

in the glycerol medium (Time 0). The regulation of the *AOX1* gene is a two step process: a repression/derepression mechanism plus an induction mechanism. Growth on glycerol derepresses transcription, and is therefore optimal for induction with methanol. Theoretically, growth on glycerol alone (before induction) is not sufficient to express low levels from the *AOX1* gene. Only methanol is able to induce detectable levels of *AOX1* expression. However, a recently successful expression of tobacco NR in *Hansenula Polymorpha* using the *MOX1* promoter also gave some expression in glycerol (personal communication). Thus, the expression of the Ricinus NR in glycerol medium could indicate that the promoter was not as tightly regulated as expected in our case.

As already mentioned, nitrate reductase also catalyses the NAD(P)H-dependent reduction of nitrite to nitric oxide (NO). Therefore, NO emission can be used as an on-line indicator of a functional NR in *Pichia* transformants *in vivo* (Figure 2.8).

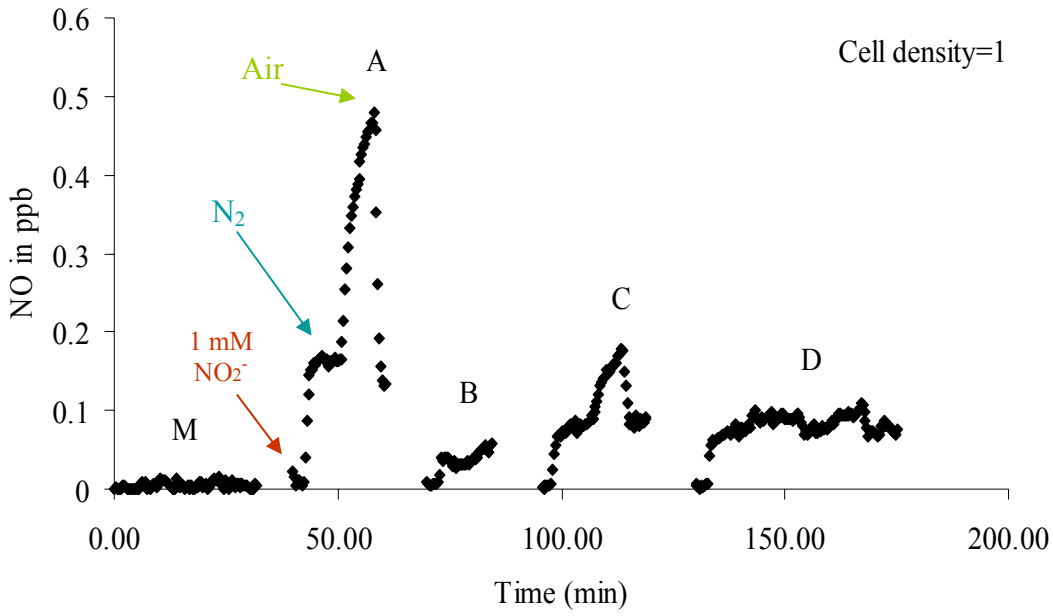

Figure 2.8 NO emission of *Pichia* transformants (*in vivo*). *Pichia* strains A, B, C and D were used to measure NO emission in the gas phase. The plasmid transformed into *Pichia* GS115 strain were pHILD2-AtNR2 (A), pPICZ-*lacZ* (B), pPICZA-RcNR (C) and pPICZA (D). M indicates the growth medium. Continuous NO-emission was measured after transfer the transformants to the induction medium for 48 h and each 1 mM nitrite was added as a substrate first in air, followed by incubation in nitrogen and subsequently by a second period in air.

Pichia cells transformed with Arabidopsis *Nia 2* gene and Ricinus NR gene produced NO right after adding of 1 mM nitrite into the cell culture. Artificial activation of leaf NR by anoxia drastically increased NO production especially in the dark (Rockel et al., 2002). When switching back from anoxia to aerobic condition, NO emission decreased again. This typical pattern for NO emission was observed with both transformants (Figure 2.8 A and C). In contrast, *Pichia* clones which expressed the *lacZ* gene or which were transformed only with the expression vector did not produce NO under the same experimental conditions. NO emission is thus an easy *in vivo* test system to check for a successful expression of functionally competent recombinant Arabidopsis NR2 and Ricinus NR.

2.3 Characterization of recombinant Ricinus NR

2.3.1 Modulation of NRs from plant leaves

NR from spinach and other plants can be rapidly inactivated *in vitro* in the presence of MgATP. This inhibition requires protein kinase and 14-3-3s. Before investigating the recombinant NRs, we first compared the ATP-dependent inactivation of NR in extracts from illuminated leaves of Arabidopsis, spinach and Ricinus (Table 1). Among these three plants, spinach NR was strongly inactivated by ATP, whereas Ricinus and Arabidopsis NR were only 9 to 25% inactivated. The initial activation state (AS) of NR in illuminated Arabidopsis and spinach leaf extracts was 70% and 80%, but only 40% or less in Ricinus (Kandlbinder et al., 2000).

Table 1. ATP-dependent inactivation of NR in extracts from illuminated leaves of Arabidopsis, Spinach and Ricinus *in vitro*. NR_{act} and NR_{max} represent the activities in the presence of either 10 mM Mg^{2+} or 10 mM EDTA. Activation state (AS) in percentage corresponds to $NR_{act} \times 100 / NR_{max}$. For further details see experimental procedures.

	Arabidopsis[a]	Spinach[a]	Ricinus[a]
NR_{act}			
–ATP	4.0 ± 0.5	9.2 ± 1.3	6.5 ± 0.8
+ATP	3.0 ± 0.5	3.0 ± 0.3	5.9 ± 1.5
NR_{max}			
–ATP	5.6 ± 0.7	11.0 ± 1.5	14.8 ± 2.3
+ATP	5.9 ± 0.7	9.4 ± 1.2	14.9 ± 2.7
AS (%)			
–ATP	71	84	44
+ATP	51	32	40

[a] Values are μmol NO_2^- per g FW per hour [± SD, n=3 (n=4 for Ricinus)]

2.3.2 Modulation of NRs from yeast cells

Having determined the response to ATP in the authentic plant NRs, Table 2 demonstrates that neither recombinant Arabidopsis NR2 (AtNR2) nor recombinant Ricinus NR (RcNR) alone could be inactivated by ATP in crude yeast extracts. This might indicate that no suitable kinases exist in *P. pastoris* extracts, because 14-3-3 proteins are common in yeasts and are able to bind to phosphorylated spinach NR *in vitro* (Moorhead et al., 1996). Next, NR-free plant leaf extracts were added to the recombinant NR's in order to add suitable protein kinase(s). AtNR2 showed about 5%, 45% and 15% of ATP-dependent inactivation in mixture with Arabidopsis darkened leaf extracts (ADL), spinach darkened leaf extracts (SDL) and Ricinus darkened leaf extracts (RDL), respectively. Moreover, NR_{act} and NR_{max} of AtNR2 were increased when mixing with NR-free extracts from leaves, but the activation states were not changed much (Table 2). In the case of the RcNR, only mixing with SDL gave a slight inhibition by MgATP. Surprisingly, the RcNR activity in the presence of Mg^{2+} (NR_{act}) was dramatically decreased by incubation with RDL (with or without ATP) but not with SDL and ADL, whereas NR_{max} remained similar. Hence, the activation state dropped remarkably only when RcNR was mixed with RDL and this low activation state was similar to that of the authentic leaf enzyme, and independent of ATP.

Table 2. Activity of recombinant Arabidopsis NR2 (AtNR2) and Ricinus NR (RcNR) in response to an incubation with MgATP (2 mM) with or without addition of desalted, NR-free leaf extracts from Arabidopsis (ADL), spinach (SDL) and Ricinus (RDL). The activity of recombinant NRs was measured in the presence of either 10 mM Mg^{2+} (NR_{act}) or 10 mM EDTA (NR_{max}). AS represents the activation state ($NR_{act} \times 100 / NR_{max}$). Data are nmol NO_2^- per mg protein per min (± SD, n=3).

	AtNR2			*RcNR*		
	NR_{act}	NR_{max}	AS (%)	NR_{act}	NR_{max}	AS (%)
–ATP	3.49 ± 0.54	4.06 ± 0.47	86	2.35 ± 0.05	2.44 ± 0.08	96
+ATP	3.62 ± 0.51	4.55 ± 0.79	80	2.47 ± 0.07	2.49 ± 0.15	99
+ADL–ATP	5.03 ± 0.34	6.60 ± 0.29	76	2.36 ± 0.15	2.48 ± 0.17	95
+ADL+ATP	4.80 ± 0.28	6.56 ± 0.37	73	2.52 ± 0.11	2.59 ± 0.13	97
+SDL–ATP	6.13 ± 0.34	7.30 ± 0.28	84	2.67 ± 0.24	2.72 ± 0.28	98
+SDL+ATP	3.37 ± 0.31	5.82 ± 1.17	60	2.27 ± 0.09	2.39 ± 0.25	95
+RDL–ATP	5.12 ± 0.75	7.18 ± 1.13	71	0.73 ± 0.40	2.11 ± 0.25	35
+RDL+ATP	4.33 ± 0.12	6.36 ± 0.24	68	0.83 ± 0.52	2.04 ± 0.28	41

2.3.3 RcNR has a high Mg^{2+}-sensitivity when mixed with NR-free extracts from Ricinus leaves at pH 7.6, but not at pH 6.5

In a previous study, nitrate reductase in extracts from hydroponically grown Ricinus leaves showed different sensitivity to pH and Mg^{2+} compared to spinach NR. NR_{act} from spinach had a broad optimum between pH 6.5 and pH 7.5, whereas NR_{act} from Ricinus had a distinct optimum around pH 6.5, and activity was very low at pH values above pH 7.3 (Kandlbinder et al., 2000). According to these findings, we also tested RcNR with regard to this unusual property. Figure 2.9 shows the time course of RcNR NR_{max} and NR_{act} alone or after addition of a desalted, NR-free leaf extract from Ricinus (RDL). In general, no Mg^{2+} inhibition of the RcNR was observed without RDL. Incubation of RcNR with RDL at pH 6.5 increased both, NR_{max} and NR_{act}. However, at pH 7.6, the mixture of RcNR plus RDL gave almost the same Mg^{2+}-sensitivity found with the authentic leaf extract. When AtNR2 was assayed under the same experimental conditions (pH 7.6), no such Mg^{2+} inhibition was observed. Taken the 10-min reaction into account, the Mg^{2+} inhibition caused by RDL incubation was about 60% (Figure 2.9B), which is consistent with the result shown in Table 2.

Apparently Ricinus leaves contain a factor which specifically interacts with RcNR, providing the properties of the native NR enzyme in leaves.

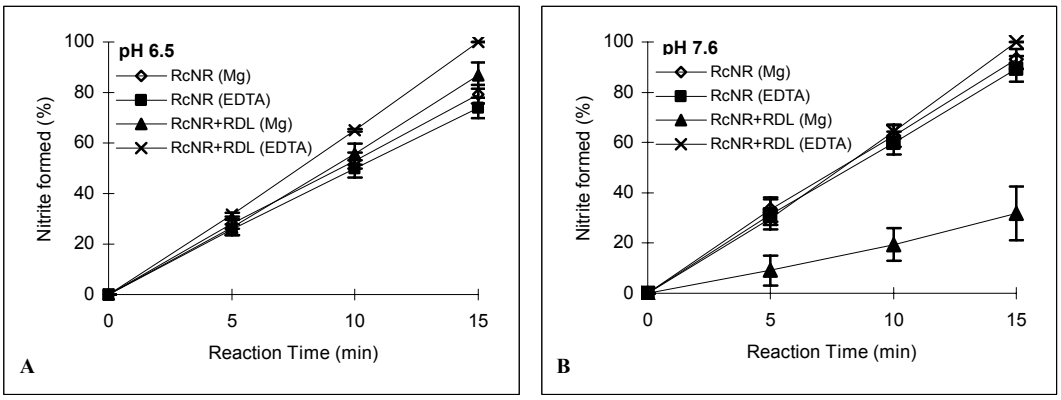

Figure 2.9 Effect of desalted crude extract from darkened Ricinus leaf (RDL) on NR_{max} and NR_{act} of recombinant Ricinus NR (RcNR). Reaction time for NR activity assays is as indicated. Vertical axis represents the nitrite formation. 100% corresponds to 22.1 and 21.5 nmol NO_2^- per mg protein for pH 6.5 (a) and pH 7.6 (b), respectively. Data are means ± SD from 3 for pH 6.5 or 4 for pH 7.6 independent experiments. More details in the text.

We further tested whether the "Mg^{2+}-sensitivity factor" was inactivated by boiling. Figure 2.10 shows that once the RDL was briefly boiled, the effect on NR_{act} was eliminated. However, the supernatant of the boiled RDL somehow activated the recombinant enzyme activities, both in Mg^{2+} and EDTA. The same phenomenon also occurred with recombinant Arabidopsis NR2, indicating that this effect was not specific for Ricinus NR. Since the supernatant of the boiled RDL contains only low molecular weight solutes, it will be interesting to find it out which component was stimulatory.

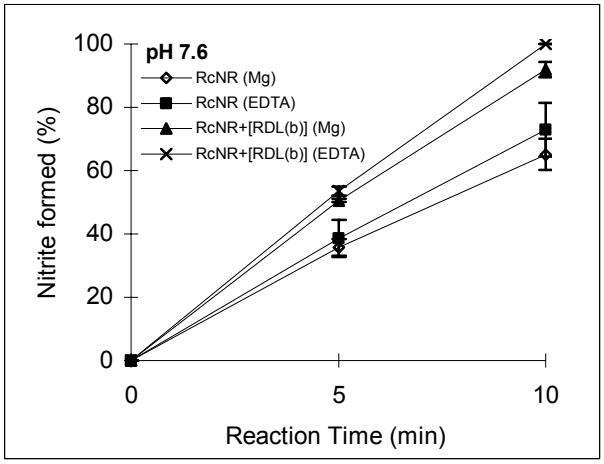

Figure 2.10 Effect of the supernatant from boiled, desalted crude extracts of NR-free Ricinus leaves [RDL(b)] on recombinant Ricinus NR (RcNR) at pH 7.6. RDL was briefly boiled (2 min) before adding into reactions. The nitrite formation was given by relating to the nitrite content of the 10-min time point of RcNR (EDTA). Reaction time for NR activity assays and the symbol for each line as indicated. The means and standard deviations of 3 replicates are given.

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2.3.4 Protein fractionation by ammonium sulfate

Ricinus leaves obviously contained one or several protein factor(s) which restored the high Mg^{2+}-sensitivity which was lost for the recombinant Ricinus NR. In a first attempt to separate such factor(s) from the crude extract, ammonium sulfate fractionation was applied providing some crude purification of proteins. A step from 0 to 35% (w/v) of ammonium sulfate (Fraction 1) indeed pulled down the protein factor(s) responsible for the high Mg^{2+}-sensitivity of RcNR (Figure 2.11). However, the remaining protein fraction precipitating between 35 to 53% ammonium sulfate

(Fraction 2) also had some effect on RcNR. Brief boiling of the proteins again eliminated the effect. Interestingly, the stimulation of RcNR activity by the supernatant from boiled RDL (without ammonium sulfate fractionation) was abolished when using the supernatants from boiled Fraction 1 and 2.

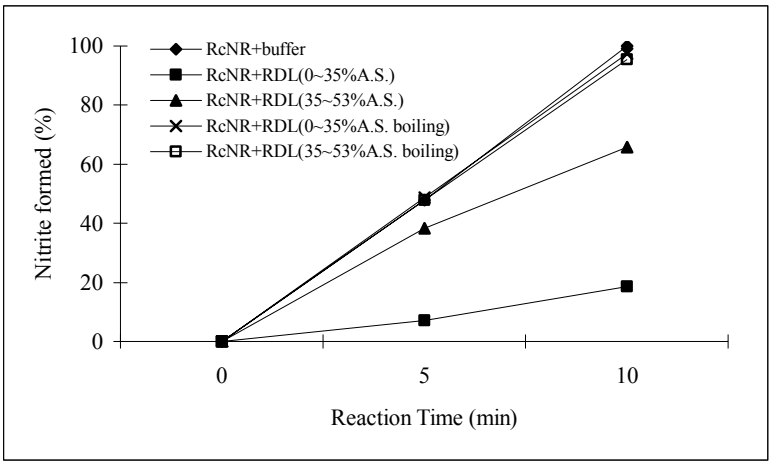

Figure 2.11 Effects of ammonium sulfate precipitation of Ricinus darkened leaf extracts on RcNR. Ricinus darkened leaf extracts (RDL) were precipitated by 0-35% (Fraction 1) and 35-53% ammonium sulfate (AS) (Fraction 2). Pellets were resuspended in the extraction buffer followed by brief boiling or without that. Protein content of two fractions was measured. About 0.7 mg of proteins from each fraction was used for each measurement. All reactions were carried out in 10 mM $MgCl_2$ for the indicated time. Data are mean values of two separate experiments.

We also checked how much of Fraction 1 was required to saturate the response of RcNR to Mg^{2+} (Figure 2.12).

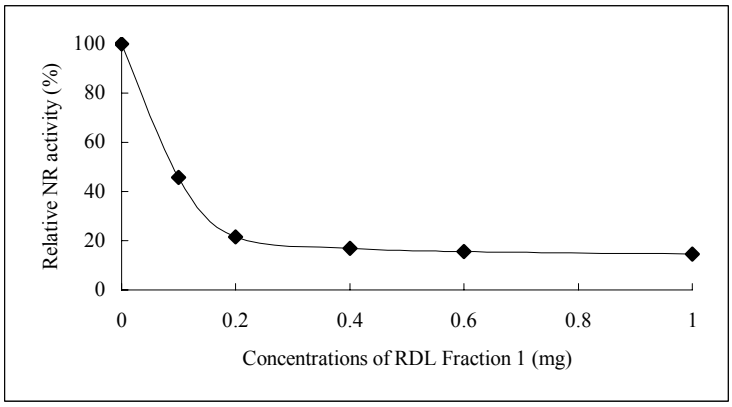

Figure 2.12 Effects of different concentrations of RDL Fraction 1 on RcNR. After 0-35% of ammonium sulfate precipitation (Fraction 1), protein was diluted in 10 mM Mg^{2+} buffer to give the concentrations indicated.

It is obvious that 0.1 mg of Fraction 1 could provide a marked inhibition of RcNR. Moreover, 0.2 mg, which is equal to 0.02% (w/v), already caused maximum inhibition.

3 Discussion

3.1 Deduced protein sequence of Ricinus NR

A multi-alignment of all available NR sequences with related proteins and enzymes revealed that 21 specific residues out of 917 in Arabidopsis NR2 were invariant and played key roles NR functions (Meyer et al., 1991; Dwivedi et al., 1994; LaBrie and Crawford, 1994; Lu et al., 1995; Su et al., 1996 and 1997; Campbell, 1999). Our sequence analysis showed that Ricinus NR shares major similarities with other plant NRs and no drastic differences were identified, apart from the N-terminal region. The 21 key residues found in Arabidopsis NR2 were entirely preserved in Ricinus NR (Appendix 6.4). However, there are some differences existing in Ricinus NR, if carefully analyzing the sequence alignment data, which might give some hints on structural features responsible for the unusual regulatory properties (compare Appendix 6.5 and 6.6).

3.1.1 The N-terminus

In spite of the disputed role of the N-terminus in NR, the comparison of this region from Ricinus NR with other higher plant NRs revealed that some interesting features (Appendix 6.5). As already mentioned, there is an acidic stretch conserved only in plant NRs. The character of this acidic stretch is two or three consecutive Ser residues followed by some Glu and Asp residues. Such a sequence is reminiscent of a consensus CK II (casein kinase II) phosphorylation site (S/TXXE/DX; Pearson and Kemp, 1991) and appears to be efficiently phosphorylated by human CK II *in vitro* (Pigaglio et al., 1999). In Ricinus NR the consensus Ser (position 58) was missing which was also absent in squash, chicory and monocots like barley, rice and maize (Appendix 6.5). Interestingly, using a program for inferring phylogenies revealed that Ricinus NR was most close to squash and chicory (discussed in 3.4). However, it is unknown whether this Ser is important for NR phosphorylation in Ricinus.

Additionally, it has been shown that a tobacco NR with an internal deletion of 56 amino acids in the N-terminal domain is no longer inactivated by MgATP (Nussaume et al., 1995). Since Ricinus leaf NR was insensitive to ATP, the variant N-terminal region might contribute.

3.1.2 The Moco-binding Region

The prosthetic Moco-binding region is located at amino acid position 92 to 321 in Ricinus NR. It has been shown that NR is phosphorylated on multiple sites (Huber et al., 1992; LaBrie and Crawford, 1994; Huber et al., 1994); therefore, the highly conserved Ser residues in this region could be sites for NR phosphorylation. Sequence comparison of the Moco domain revealed that Ricinus NR possesses those highly conserved Ser residues (Ser211, 253, 258, 316, 357, 387 and 430) as Arabidopsis NR2 does. A recent study on site-directed mutagenesis of these Ser residues of *Pichia*-expressed Arabidopsis NR2 showed that none of these Ser residues are essential for the *in vitro* inhibition (Su et al., 1997). On the other hand, Ricinus NR did contain some unique amino acid residues when compared with other plant NRs (Appendix 6.6). For example, there is a His103 (basic amino acid residue) in Ricinus NR where all other NRs have aspargine (polar amino acid with uncharged R group) instead. In addition, Gln123 (others: Arg), Cys157 (most of others: Thr), Asn212 (others: Thr), Val266 (others: Asp) and Ala284 (others: Pro) were found to be the specific residues in Ricinus NR. These amino acid residues might have an influence on the incorporation of Moco into the prosthetic domain. To date, very little is known about how Moco becomes incorporated into NR. One crystallograohic analysis of the Mo-enzyme suggests that the cofactor is deeply buried within the holo-enzyme so that Moco could only have been incorporated prior to or during completion of folding and dimerization of the apoprotein (Kisker et al., 1997; Enroth et al., 2000). Nevertheless, more investigations are required in order to find out whether these amino acid residues also play a role in the regulation of Ricinus NR.

3.1.3 The Hinge 1 Region

The potential 14-3-3 protein binding motif is located in Hinge 1 region of NR. It has been reported that the 14-3-3 protein binds directly to the regulatory phosphorylation site on phospho-NR (Bachmann et al., 1996c). Therefore, either the compounds which can interact with 14-3-3s such as divalent ions or AICAR or the sequence expanded outside the 14-3-3 binding region may influence 14-3-3 binding (Athwal et al., 1998ab). If numbering the Ser526 of Ricinus NR as the position 0, the sequence alignments revealed that Ricinus NR has an alanine (Ala540) at position +14 where all other NRs have a serine residue (Appendix 6.6). In addition to that, the amino acid residues at minus positions (up to -18) also showed a possible role in the binding of

NR to the 14-3-3 protein (Athwal et al., 1998b). We found that an alanine (Ala498) instead of glycine positions at -28 is unique in Ricinus NR.

Taken together, the sequence within this region of Ricinus NR showed some interesting characters. So far, we have no evidence whether 14-3-3 proteins indeed interact with NR in Ricinus. With respect to the observations mentioned above, it is likely that the interaction between Ricinus NR and 14-3-3s is different from other NRs.

3.2 Heterologous expression of Ricinus NR

Expression of full-length Ricinus NR in *Pichia pastoris* resulted in a functional enzyme. However, the levels of protein produced were not detected in a Coomassie-stained SDS-PAGE. As a matter of fact, we always observed strong signals in the lower molecular weight region (70-80 kDa). This could be caused by inappropriate treatment of protein samples, or recombinant RcNR itself was protease sensitive. Interestingly, in the Hinge 1 region of Ricinus NR a residue Arg482 which provides an additional predicted Trypsin cleavage site within ^{481}KRHK^{484} (while most of plant-NRs possess KPHK) was found (Appendix 6.6). On the other hand, in recent work on heterologously expressed tobacco NR in the yeast *Hansenula polymorpha* the recombinant protein was also not detected on SDS-PAGE (Perdomo et al., 2002). This may indicate that only low levels of plant NRs were produced in yeasts. Moreover, the activities obtained *in vitro* from the recombinant Ricinus NR (RcNR) and the same system expressing Arabidopsis NR2 (AtNR2) were 2 to 4 nmol nitrite per mg protein per min, which are also very similar to tobacco NR expressed in a nitrate assimilatory yeast, *Hansenula polymorpha* (Perdomo et al., 2002).

3.3 Characterization of recombinant RcNR

3.3.1 *In vitro* inactivation by ATP

One of the unusual characters of authentic Ricinus NR was its insensitivity to preincubation with ATP (Kandlbinder et al., 2000), which contrasted sharply with the situation in spinach and other plants (Kaiser and Spill, 1991; Huber et al., 1994). It has been shown previously that under continuous darkness for up to 6 days, spinach NR protein was almost completely degraded. However, NR kinase(s), NR phosphatase(s) and 14-3-3 proteins were still present (Glaab and Kaiser, 1996). Mixing AtNR2 (which, by itself, was not inactivated by preincubation with ATP) with

a NR-free spinach leaf extract (4-day darkness) resulted in significant inactivation of AtNR2 with ATP. In contrast, RcNR was never inactivated with ATP, neither after addition of Arabidopsis darkened leaf extract nor darkened leaf extract from Ricinus.
As deduced from the sequencing data, Ricinus NR has a normal phosphorylation site in hinge 1. Consequently, lack of ATP-inactivation in a mixture of RcNR and leaf extracts may either reflect absence of a specific protein kinase activity, or a lack of specific 14-3-3 proteins. It has been shown previously that Ricinus leaves do contain 14-3-3s (Kandlbinder et al., 2000), and so do yeast cells (Van Heusden et al., 1992; Gelperin et al., 1995). Addition of recombinant yeast 14-3-3s (up to 20 μg per mL), which efficiently inactivates phospho-NR from spinach (Moorhead et al., 1996), partially purified NR from spinach, transgenic tobacco plants and squash (Lillo et al., 1997) and Arabidopsis cell extracts (Moorhead et al., 1999) did not at all affect RcNR activity (not shown). It seems obvious that the insensitivity to ATP is not due to the absence of the regulatory phosphorylation site nor to a lack of 14-3-3 proteins, but to some as yet unidentified structural deviations in the less well conserved parts of the protein, i.e. N-terminal domain. Therefore, the conclusion is that this ATP-insensitivity is an inherent property of Ricinus NR.

3.3.2 Specific protein factor(s) for high Mg^{2+}-sensitivity of RcNR

In crude yeast extracts, RcNR lacked the high Mg^{2+}-sensitivity typically observed for NR in crude extracts from Ricinus leaves at pH 7.3, and was not much different in that respect from AtNR2 (Table 2). Loss of the unusual Mg^{2+}-sensitivity of Ricinus NR might have two reasons: i) factors contained in the yeast extract might interact with NR to render it Mg^{2+} insensitive, or ii) the yeast extracts lacked factors required for the high Mg^{2+}-sensitivity.
Our data suggest that the latter explanation is correct. Leaves from Ricinus, as from spinach, can be made virtually NR free by exposing them to continuous darkness for 4 days. Addition of such Ricinus extracts from darkened leaves (RDL) almost fully restored the high Mg^{2+}-sensitivity of the authentic leaf enzyme. Boiling of the extracts prior to addition prevented the restoration of Mg^{2+}-sensitivity, indicating that the required factor was probably a protein. This was further confirmed by using ammonium sulfate to successfully precipitate the factor(s). In conclusion, the unusual high Mg^{2+}-sensitivity of Ricinus NR appears not an inherent property of the NR

protein itself. Rather, it is the consequence of an yet unidentified protein contained in Ricinus leaves that interacts with NR.

We do not know whether Ricinus NR and the unidentified protein are in contact within the intact leaf cells, or whether they are separated by subcellular compartmentation. In the latter case, the high Mg^{2+}-sensitivity of Ricinus NR observed in leaf extracts would represent an artifact due to homogenization of the leaf cells for extraction. But on the other hand, addition of RDL to AtNR2 did not increase the Mg^{2+}-sensitivity of the latter. Thus, the interaction of the above-mentioned protein factor from Ricinus leaves with RcNR appeared specific for RcNR, pointing to the probability of a natural, non-artificial interaction.

3.3.3 Possible regulatory factors of NR

Incubation of AtNR2 with different NR-free leaf extracts gave a significant activation of the enzyme activities, both in Mg^{2+} and EDTA. This could be due to either a general positive factor for NR existing in leaves, or recombinant Arabidopsis NR2 may contain special features in its sequence. However, such activation was not observed in RcNR (Table 2). If the first assumption is correct, in case of RDL, the disappearance of the positive effect in RcNR might be somehow counterbalanced by the negative factor(s) mentioned above. Mixing RcNR with ADL and SDL, however, should give the same activation, when the positive factor is common in plant leaves. Therefore, it seems that the second speculation is more likely, that Arabidopsis NR2 possesses special structural characters. Nevertheless, it is also likely that Ricinus NR does not interact with that common factor, if there is any, because of its yet unidentified structural difference from other NRs.

In addition to that, Ricinus leaf extracts apparently contained non-protein components that were able to increase both, NR_{max} and NR_{act} of RcNR. It is yet unclear whether these components would abolish potentially negative effects of yeast compounds, or if they would directly interact with NR. The nature of these compounds is also unknown at present.

Altogether, the unknown protein factor which renders RcNR Mg^{2+}-sensitive is most probably not a 14-3-3 protein, nor appears the high Mg^{2+}-sensitivity related to NR protein phosphorylation. In summary, our original conclusions that Ricinus NR has some unusual properties are confirmed by the above presented data. Additionally, Ricinus leaf cells contain a protein factor which appeared to interact specifically with

Ricinus NR to provide the unusual pH- and Mg^{2+}-response. More investigations are required to identify the structural components in the RcNR protein that are responsible, to identify the protein factor and also to elucidate the physiological background and consequences of these extraordinary regulatory properties.

3.4 The comparison of Ricinus and squash nitrate reductase

Since we observed the unusual regulatory properties from Ricinus NR, one question arises regarding the physiological meaning of the specific properties of Ricinus NR. We are still not able to answer this question from our present data. From the evolutionary point of view, however, to figure out the phylogenies of Ricinus NR might give some hints. Using PHYLIP package to compare 18 NADH-specific nitrate reductases revealed that Ricinus NR was most close to squash NR (*Cucurbita maxima*) with respect to both full-length amino acid sequence and N-terminus comparisons (Appendix 6.7 and 6.8).

Squash NR was first cloned in 1986 and a protein of 918 amino acids was encoded (Crawford et al., 1986; Hyde et al., 1991). Some studies in squash NR were done by Lillo and coworkers. They found that squash NR can be activated by substrates, a so called hysteretic behavior (Lillo and Ruoff, 1992). Two forms of NR, high and low activity forms, existing in squash crude extracts and purified enzyme in response to light-dark transition. The light form (high activity) was not inhibited by Ca^{2+} and Mg^{2+}. The dark form (low activity) was in contrary strongly inactivated by both divalent cations (Lillo, 1993 and 1994). NR activity in crude extracts from light-exposed plants or partially purified NR could be inhibited by Mg^{2+} after foregoing incubation with MgATP. Therefore, the inhibition by Mg^{2+} or Ca^{2+} was related to phosphorylation or a phosphorylated form of NR (Kaiser and Spill, 1991; Huber et al., 1992; MacKintosh, 1992). However, squash NR can be converted to a form susceptible to Ca^{2+} and Mg^{2+} inhibition without preincubation with MgATP. Hence, for squash, no MgATP was necessary for converting the high activity form of the enzyme into low activity form. Assumption of a phosphorylation process resulting in decreased NR activity was irrelevant for squash NR.

Furthermore, in the absence of Mg^{2+} the increase of NR activity of squash crude extracts by substrates was observed when made from dark-exposed leaves but not from leaves in the light. Partially purified squash NR, however, was found no

difference between NR extracted from leaves in the dark and light with respect to substrate activation (Lillo et al., 1997).

Taken together, their conclusion was that an additional way of regulating NR independently of the phosphorylation state of the enzyme might exist. It was interesting that purified squash NR differed from the enzyme in crude extracts. Does that point to the existence in crude extracts of a possible regulatory factor for squash NR, similar as described above for RcNR? In summary, it may seem that Ricinus NR and squash NR share similar regulatory properties which appear in some way different from the common post-translational modulation. Certainly, it would be interesting to compare both enzymes in more details, especially under the same experimental conditions.

4 Materials and Methods

4.1 Plant Material

Spinach (*Spinacia oleracea* L. cv. Polka F1) was grown in soil culture in the greenhouse. The mean daylength was 11 h with supplementary illumination (HQi, 400W; Schreder, Winterbach, Germany) at a total photon flux density of 250 to 400 μmol m^{-2} s^{-1} photosynthetically active radiation. Air humidity varied from 60 to 80%, and day/night temperature from 20 to 26°C and 16 to 22°C. The plants were fed with a commercial nitrate fertilizer.

Seeds of Ricinus (*Ricinus communis* L.) were germinated in soil and grown in the greenhouse under the conditions mentioned above. Plants were used for experiments after about 5 weeks (if not mentioned otherwise).

Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) was grown in soil (Type P, Gebr. Hagera Handelsges.m.b.H., Sinntal-Jossa, Germany) and exposed to white light for 8 h per day at 160 μmol m^{-2} s^{-1}.

4.2 RNA Protocols

4.2.1 Total RNA Isolation

Total RNA, for use as a template for cDNA synthesis, was isolated from the leaf tissue of nitrate-treated Ricinus plants using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The procedure is based on the selective binding properties of a silica-gel-based membrane in a microspin column system. A specialized high-salt buffer system allows the binding of up to 100 μg of total RNA longer than 200 bp to the membrane.

Leaf material was ground in the nitrogen to a fine powder using a mortar and pestle. 100 mg of tissue powder was mixed with 900 μL Lysis buffer. The lysate was applied to a QIAshredder spin column and centrifuged for 2 min at maximum speed twice. The flow-through fraction was mixed with 450 μL of ethanol and transferred to an RNeasy spin column for absorption of RNA to membrane. After several washing and brief centrifugation the RNA was eluted in 30 μL DEPC-treated H_2O twice. The concentration of RNA was read in a spectrophotometer (GeneQuqnt II, Pharmacia

Biotech) by measuring the absorbance at 260 nm. A ratio between A_{260} and A_{280} with a value of 2 was used as a criterion for pure preparation of RNA. The RNA was stored at -80°C.

4.2.2 Electrophoresis of RNA

Total RNA was used for a denaturing electrophoresis using formaldehyde-agarose gels. Formaldehyde forms unstable Schiff bases with the single imino group of guanine residues. These adducts maintain RNA in the denatured state by preventing intrastrand Watson-Crick base pairing.

4 µL of formaldehyde gel-loading buffer was added to 6 µg total RNA and incubated at 65°C for 10 min. RNA samples were chilled for 2 min on ice and loaded in a formaldehyde-agarose gel. After electrophoresis of RNA (at 80V) in the presence of ethidium bromide, the 28S and 18S species of rRNA should be visible under UV illumination (ImageMaster VDS, Pharmacia Biotech). Distinct clear bands and the 28S band should be stained at approximately twice the intensity of the 18S band prove the quality of the preparation.

10× MEN-buffer:

0.2 M MOPS
0.01 M EDTA
0.05 M Sodium acetate

Gel loading buffer:

720 µL Formamide
160 µL 10× MEN
260 µL 37% Formaldehyde
180 µL H_2O
100 µL 80% Glycerol
80 µL 2% Bromophenol blue
3 µL 1% Ethidium bromide

Formaldehyde-agarose gel (30 mL):

0.3 g Agarose
3 mL 10× MEN
25.5 mL DEPC-treated H_2O
(Dissolved in the microwave, cooling to 50°C)
1.62 mL 37% Formaldehyde

4.3 DNA Protocols

4.3.1 Isolation of Plasmid-DNA from *Escherichia coli*

Plasmid DNA isolation is based on alkaline lysis of bacterial cells followed by selective precipitation of genomic bacterial DNA and proteins by lowering the pH.

1.5 mL of overnight grown plasmid containing *E. coli* culture in LB was harvested and lysed with 300 µL of P1 buffer. After addition of 300 µL of P2 buffer and gentle mixing by inversion, the mixture was incubated at RT for 3 min. Subsequently, 300 µL of chilled P3 buffer were added, mixed and incubated on ice for 10 min. The cell debris and chromosomal DNA were precipitated with SDS and potassium acetate. After pelleting the debris the plasmid DNA was precipitated from the supernatant with 600 µL isopropanol. The DNA pellet was washed in cold 70% ethanol. After careful and complete removal of ethanol, the DNA was air-dried and then resuspended in TE buffer. The concentration of DNA was determined by UV spectroscopy at 260 nm. A ratio between A_{260} and A_{280} with a value of 1.8 was used as a criterion for pure preparation of DNA. The plasmid-DNA was stored at -20°C.

LB medium: 1% Tryptone; 0.5% Yeast extract; 1% NaCl; pH 7.0.
P1: 50 mM Tris-HCl pH 8.0; 10 mM EDTA; 100 µg/ml RNase A
P2: 200 mM NaOH; 1% SDS
P3: 3 M Potassium acetate pH 5.5
TE buffer: 10 mM Tris-HCl pH 8.0; 1 mM EDTA

4.3.2 Restriction Endonucleases Digestion

In most cases two units of enzyme were used to completely digest 1 µg plasmid DNA during 1 h at 37°C. In the case of *Sac* II and *Apa* I double cleavage, buffer B+ (MBI Fermentas) was used for optimal condition (maximum activity) of both enzymes. Addition of 1/5 volume of loading buffer to stop the reaction and the digested mixture was subjected to electrophoresis.

4.3.3 Electrophoresis of DNA

Agarose gel electrophoresis is the easiest and most common way of separating and analyzing DNA. Most agarose gels are made between 0.7% and 2%. A 0.7% gel will show good separation (resolution) of large DNA fragments (5 to 10kb) and a 2% gel will show good resolution for small fragments (0.2 to 1kb). Routinely, 1% gels which

are suitable for a molecule range of 0.5 to 12 kb were used. The agarose was melted in 1× TAE and mixed with 0.2 µg/mL ethidium bromide after cooling to 50°C. The DNA sample was mixed with an appropriate amount of loading buffer and subjected to electrophoresis, performed in a 1× TAE buffer system at a voltage of 5 to 10 V/cm. 1 kb Ladder was used as molecule size marker (Gibco BRL, Eggenstein, Germany). The DNA could be checked by the fluorescence of the DNA-intercalating dye ethidium bromide under UV illumination.

50× TAE buffer: 2 M Tris-Acetic acid pH 8.0; 50 mM EDTA pH 8.0
Loading buffer (6×): 50% Glycerol; 7.5 mM EDTA; 0.4% Xylenxyanol; 0.4% Bromophenol blue

4.4 Sequencing

Sequence information was obtained by the Sanger-Coulson method using the "Thermo Sequence fluorescent labeled primer cycle" sequencing-kit with 7-deaza-dGTP" (Amersam Pharmacia Biotech, Freiburg, Germany). Software for the sequencing was Base Imagir 4.0 (L1-COR, MWG-Biotech GmbH, Ebersberg, Germany). To compare the new sequences with already known entries the BLAST-Algorithms (Altschul *et al*., 1990) of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) were used. ClustalX and Genedoc software was used for sequence alignments.

4.5 Molecular Cloning of Ricinus NR by RT-PCR

RT-PCR (reverse transcription-polymerase chain reaction) was used for cloning the NR gene from Ricinus. The technique consists of two parts: synthesis of cDNA from RNA by reverse transcription (RT) and amplification of a specific cDNA by polymerase chain reaction (PCR). The first task when using RT-PCR for mRNA analysis is RNA isolation. The RNA should be high quality and free from genomic DNA contamination. Every RT-PCR begins with a reverse transcriptase reaction. The RT reaction uses a RNA template (typically either a total or poly(A) RNA sample), a primer (random or oligo dT primers), dNTPs, buffer and a reverse transcriptase (M-MLV or AMV RT). After synthesis of the first-strand cDNA, a pair of specific primers is then used to amplify the target region via PCR.

PCR is a powerful technique that allows amplifying a specific DNA sequence millions of times in a few hours. During PCR, there are three basic steps. First, the target genetic material must be denatured, that is, the strands of its helix must be unwound and separated by heating to 90-96°C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the now single-stranded DNA. The third is DNA synthesis by a polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides very quickly. The result are two new helixes in place of the first, each composed of one of the original strands plus its newly assembled complementary strand.

A Ricinus partial NR cDNA was amplified by RT-PCR. For reverse transcription a 18mer oligo-dT oligonucleotide was used as the 3'primer. cDNA was synthesized from 6 µL of total RNA (c.a. 1 µg mRNA) with SuperScript^{TM} II RNase H^{-} MMLV Reverse Transcriptase (Gibco BRL, Eggenstein, Germany) using the procedure detailed in the RETROscript^{TM} kit for the first-strand synthesis (Ambion).

20 µL reaction:

1 µg mRNA
1 µL oligo dT_{18} (50 µM)
4 µL H_2O

Mix the above components and heat the mixture at 70°C for 10 min. Remove tube(s) to ice; spin briefly, replace on ice. Subsequently add

4 µL 5× first strand buffer
2 µL DTT (0.1 M)
2 µL dNTPs (5 mM each)

Mix gently, spin briefly. Incubate the mixture at 37°C for 2 min for equilibration. Then add 1 µL (200 units) of SuperScript RT and incubate at 42°C for one hour.

For PCR amplification an oligonucleotide corresponding to the conserved sequence 5'-ATTGGTGGAAGAATGGTAAAGTGG matching positions 898 to 921 of the potato *Nia* gene coding sequence (Harris et al., 2000) was selected as a 5' gene-specific primer. A 21mer oligonucleotide 5'-CACGAACAATCTCTTTGGCAC corresponding to the Arabidopsis *Nia1* gene at positions 1343 to 1363 was used as the 3'-primer. The component of the reaction was as the followings:

50 µL reaction:

1 µL RT reaction (see above)
5 µL 10× PCR buffer
2.5 µL $MgCl_2$ (50 mM)

2.5 µL dNTPs (5 mM each)
1 µL 5' gene-specific primer (20 µM)
1 µL 3'-primer (20 µM)
0.2 µL *Taq* DNA polymerase (5U/µL)
36.8 µL dH_2O

Amplification was performed using an annealing temperature of 55°C. The cycling parameters were (a) heat 5 min., 94°C; (b) cycle 30: 94°C, 30 sec.; 55°C, 30 sec.; 72°C, 30 sec.; (c) hold 5 min., 72°C. It produced a cDNA fragment of the expected size (ca. 440 bp based on the positions of the oligonucleotide sequence in the potato *Nia* gene sequence) which was subcloned into the pCR® 2.1-TOPO vector (Invitrogen, Groningen, the Netherlands) for DNA sequencing.

4.6 Amplification of full-length Ricinus NR cDNA by RACE

Following reverse transcription, the first-strand cDNA can be used in 5'-RACE and 3'-RACE (Rapid Amplification of cDNA Ends). However, it is important to ensure that the first-strand cDNA reaches the end of an RNA template. This is achieved by using MMLV reverse transcriptase (RT). When certain MMLV RT variants reach the end of an RNA template, they exhibit a terminal transferase activity that adds 3-5 residues (predominantly dC) to the 3' end of the first-strand cDNA (Figure 4.1).

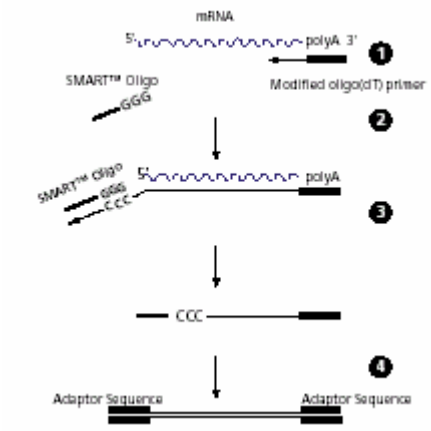

Figure 4.1 Mechanism of SMART cDNA synthesis. (1) First-strand synthesis is primed using a modified oligo(dT) primer. (2) After reverse transcription reaches the end of the mRNA template, it adds several dC residues. (3) The SMART II oligonucleotide anneals to the tail of the cDNA and serves as an extended template for RT. After RT switches templates from the mRNA molecule to the SMART oligo, a complete cDNA copy of the original RNA is synthesized with the additional SMART sequence at the end. (4) The complete first-strand cDNA then would have an adaptor sequence at both ends.

In the SMART technology (BD Biosciences Clontech, Heidelberg, Germany), this activity is harnessed by the SMART oligo whose terminal stretch of dG residues can anneal to the dC-rich cDNA tail and serve as an extended template for RT. After RT switches templates from the mRNA molecule to the SMART oligo, a complete cDNA copy of the original RNA is synthesized with the additional SMART sequence at the end. Since the dC-tailing activity of RT is most efficient if the enzyme has reached the end of the RNA template, the SMART sequence is typically added only to complete first-strand cDNAs. This process guarantees that the use of high quality RNA will result in the formation of a set of cDNAs that has a maximum amount of 5' sequence. The only requirement for SMART RACE cDNA amplification is that at least 23-28 nucleotides of sequence information is known in order to design gene-specific primers for the 5'- and 3'-RACE reactions.

The full-length Ricinus NR cDNA was obtained by following the manufacturer's instructions (BD Biosciences Clontech, Heidelberg, Germany). 6 μg of total RNA were used for preparation of either SMART^{TM} first-strand cDNA synthesis (5'-RACE-Ready cDNA) or standard first-strand cDNA synthesis (3'-RACE-Ready cDNA), depending on the presence of SMART II oligo (5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3') in the reaction or not. After generating RACE-Ready cDNAs, 5'-RACE and 3'-RACE were performed with the Advantage® 2 Polymerase Mix (BD Biosciences Clontech, Heidelberg, Germany). PCR reaction was performed by using the gene specific primers 5'-TCTCTTTGGCACCAAGGAGGTCCAG-3' for 5'-RACE and 5'-GCTGAAGCATGGTGGTATAAGCCGG-3' for 3'-RACE, respectively. DNA was amplified by 25 cycles of 94°C for 10 sec, 68°C for 20 sec and 72°C for 3 min in a PCR-reaction.

4.7 *Pichia pastoris* Expression System

Yeast hosts that can be used for expression studies *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, and *Yarrowia lipolytica*. The first three are the most widely used. Yeasts are particularly attractive as expression hosts for a number of reasons. They can be rapidly grown on minimal (inexpensive) media. Recombinants can be easily selected by complementation, using any one of a number of selectable

(complementation) markers. Expressed proteins can be specifically engineered for cytoplasmic localization or for extracellular export. And finally yeasts are exceedingly well suited for large-scale fermentation to produce large quantities of heterologous protein.

The methylotrophic yeasts, *H. polymorpha* and *P. pastoris*, are unique in that they will grow using methanol as the sole carbon source. In P. pastoris, growth in methanol is mediated by alcohol oxidase, as enzyme whose de novo synthesis is tightly regulated by the alcohol oxidase promoter (*AOX1*). The enzyme has a very low specific activity. To compensate for this, it is overproduced, accounting for more than 30 percent of total soluble protein in methanol-induced cells. Thus, by engineering a heterologous protein gene downstream of the genomic *AOX1* promoter, one can induce its overproduction.

Ricinus NR cDNA was inserted into the *Sac* II and *Apa* I site of the *Pichia pastoris* expression vector pPICZA (Invitrogen). The expression clone was transformed into *P. pastoris* strain GS115 by EasyComp™ Transformation, as described by EasySelect™ *Pichia* Expression Kit (Invitrogen, Groningen, the Netherlands).

Growth media and conditions for selection of transformants were as described by the manufacturer (Invitrogen). Zeocin^R transformants were selected. Cells expressing NR protein were identified as follows: To induce NR gene expression, which was driven by the alcohol oxidase promoter of *P. pastoris,* transformants were first grown in modified BMG(H) (Su et al., 1997) (1.34% [w/v] YNB, 1% [v/v] glycerol, 100 mM potassium phosphate, pH 6.0, 4×10^{-5}% biotin, 0.2 mM sodium molybdate and w/o 4×10^{-5}% histidine) to an OD_{600} of 2 to 6. Cells were centrifuged and then resuspended in modified BMM(H) medium (1.34% [w/v] YNB, 0.5% [v/v] methanol, 100 mM potassium phosphate, pH 6.0, 4×10^{-5}% biotin, 0.2 mM sodium molybdate and w/o 4×10^{-5}% histidine) at OD_{600} of 1 and grown for up to 72 h. Methanol (0.5% [v/v]) and sodium molybdate (0.2 mM) were added to the culture every 24 h. After a 48 h induction period the cells were collected by centrifugation to OD_{600} of ~20, frozen quickly in liquid N_2 and stored at -80°C until ready to assay.

4.8 Protein Protocols

4.8.1 Protein Determination

The protein content of the samples was determined with BCA reagent (Pierce, Rockford, Ill, USA) and Bovine serum albumin (BSA) as a standard which is based on the method of Smith et al., 1985. Using the reaction of copper(II) with protein and the subsequent reaction of BCA with copper (I), a colormetric compound is formed which is quantified by spectroscopy.

4.8.2 SDS-Polyacrylamide Gelelectrophoresis (SDS-PAGE)

Protein separation by SDS-PAGE is used to determine the relative abundance of major proteins in a sample, their approximate molecular weights, and in what fractions they can be found. The purity of protein samples can be assessed. Different staining methods can be used to detect rare proteins.

Pichia protein samples were obtained by phenol extraction/ammonium acetate precipitation. The samples were boiled in sample buffer (Roti®-Load 1; Roth, Karlsruhe, Germany) containing 2-mercaptoethanol which is a mild reducing agent for cleaving disulfide bonds to thiols, and SDS, an anionic detergent used for denaturation of native proteins. 20 µg of the protein solution were loaded onto the polyacrylamide gel and separated at constant 30 mA. As protein standards the 10 kDa Protein Ladder (Gibco BRL) was used. Compositions of the gel and buffers were as follows (Neville, 1971):

Resolving gel: 14 mL of two 10% gels

3.6 mL 40% Acrylamide/bisacrylamide (29:1 mix)
2.8 mL Tris-HCl pH 9.18
0.14 mL 10% SDS
7.35 mL H_2O
6.25 µL TEMED
22 µL 40% Ammonium persulfate

Stacking gel: 10 mL of 4 gels

1.5 mL 40% Acrylamide/bisacrylamide (29:1 mix)
2.5 mL Tris-H_2SO_4 pH 6.1
0.1 mL 10% SDS
5.8 mL H_2O
10 µL TEMED
25 µL 40% Ammonium persulfate

Running buffers:
Lower: 424 mM Tris-HCl pH 9.18
Upper: 41 mM Tris-Boric acid pH 8.64; 0.1% SDS

4.8.3 Western Blot and Immunodetection of NR

For transfer of proteins onto a nitrocellulose membrane (Schleicher und Schüll, Dassel, Germany) a semi-dry system (Biometra, Göttingen, Germany) was used. The transfer was performed at 0.8 mA/cm^2 for 1 h.

Anode (Top)
3× Whatman 3MM filter paper/Anode buffer 1
3× Whatman 3MM filter paper/Anode buffer 2
Transfer membrane/Anode buffer 2
SDS-PAGE gel
3× Whatman 3MM filter paper/Cathode buffer
Cathode (Bottom)

Cathode buffer: 40 mM 6-Aminocapronic acid; 20% Methanol
Anode buffer 1: 0.3 M Tris pH 8.8; 20% Methanol
Anode buffer 2: 25 mM Tris pH 8.8; 20% Methanol

For NR protein detection the immunoenzymatic staining method was used. The membrane with the bound proteins was further treated as follows:

Process	Buffer	Time
Blocking	5% milk powder in 1× TBS; 0.1% Tween 20	1 h at RT
Washing	1× TBS; 0.1% Tween 20	2× 5 min
Primary antisera incubation	1:2000 in 1× TBS; 0.1% Tween 20	1 h at RT
Washing	1× TBS; 0.1% Tween 20	3× 5 min
2nd antibody-AP incubation	1:3000 in 1× TBS; 0.1% Tween 20	45 min at RT
Washing	1× TBS; 0.1% Tween 20	3× 5 min
Staining	AP conjugate substrate kit	5-30 min

2nd antibody: Alkaline phosphatase (AP) labeled goat anti-rabbit IgG
(Southern Biotechnology Associates, USA)
AP conjugate substrate kit: AP color reagent A and B; development buffer
(Bio-Rad, München, Germany)

4.8.4 Ammonium Sulfate Fractionation

Proteins are usually soluble in water solutions because they have hydrophilic amino acids on their surfaces. Increasing the salt concentration to a very high level will cause proteins to precipitate from solution without denaturation if done in a gentle manner. When salt, such as ammonium sulfate, is added to the protein solution, the salt ions

attract the water molecules away from the protein; hence, the protein molecules are forced to interact with themselves and begin to aggregate. If this is carried out on ice, the proteins will precipitate without denaturation. Thereafter, the proteins can be collected by centrifugation and then redissolved in solution using a buffer with low salt content. All procedures have to be carried out at 4°C. Ammonium sulfate is the most common salt used for this purpose because it is usually soluble in cold buffer.

9 g of 4-day darkened Ricinus leaves were ground in liquid nitrogen and 3-fold (27 mL) volume of extraction buffer (content see 4.9.2) was added to the fine powder. After grinding, the mixture was centrifuged at 14500 rpm for 15 min. The Supernatant was then filtered through a paper filter. Following the determination of the exact volume of the supernatant, the amount of ammonium sulfate corresponding to 35% (w/v) was slowly added to the supernatant while the latter was continuously stirred. The mixture was kept on a magnetic stirrer for additional 20 min prior to centrifugation at 14500 rpm for 15 min. After centrifugation, the pellet was resuspended in extraction buffer (Fraction 1) and kept on ice. The supernatant was used for further precipitation by adding an additional amount of ammonium sulfate to 53% (w/v). Stirring and centrifugation procedures were repeated. The pellet was again resuspended in extraction buffer (Fraction 2), and kept on ice. The protein concentration of the two fractions was measured (see 4.8.1).

4.9 NR activity Measurements

4.9.1 Determination of NR activity in yeast cells (*in vivo*)

Pichia transformants were tested for their NR-expression levels. Aliquots of the cell culture were taken every 24 h during a 72 h induction period. The NR activity was measured after suspending *Pichia* transformants (OD_{600}=1) in 1 mL of 100 mM HEPES buffer pH 7.3 containing 50 mM KNO_3. After a 30-min incubation at 28°C, nitrite released to the medium was determined as described below.

4.9.2 Assay of NR activity *in vitro*

For plant NRs: Leaf material from Ricinus and Arabidopsis was ground in liquid nitrogen and 1.5 mL (1.0 mL for Arabidopsis) of extraction buffer (100 mM HEPES, pH 7.6, 5 mM DTT, 10 mM $MgCl_2$, 10 μM FAD, 10 μM sodium molybdate, 50 μM

Leupeptin, 2 mM Pefabloc, 0.02% casein, 0.5% polyvinylpolypyrolidone and 0.05% BSA) was added to 0.5 g FW. In the case of spinach, 1.0 mL of extraction buffer (without 0.02% casein, 0.5% polyvinylpolypyrolidone and 0.05% BSA) was added to 0.5 g FW. After grinding, the suspension was centrifuged at 13000 rpm for 12 min at 4°C. The supernatant was desalted on Sephadex G25 spin columns (1.6 mL resin volume for 650 µL extract, 4°C) equilibrated with extraction buffer.

For recombinant NRs: Frozen yeast cells (OD_{600} of ~20) were thawed on ice and resuspended in 500 µL extraction buffer containing 100 mM HEPES, pH 7.6, 5 mM DTT, 10 mM $MgCl_2$, 0.3% Triton X-100, 10 µM FAD, 10 µM sodium molybdate, 50 µM Leupeptin, 2 mM Pefabloc and an additional protease inhibitor cocktail (Roche, Mannheim, Germany). An approximately equal volume of glass beads (450 to 600 µm; Sigma) were added. The mixture was vortexed 8 times using 1-min bursts followed by 1-min cooling periods. The crude extract was centrifuged at 13000 rpm for 12 min at 4°C.

The following assays were carried out with aliquots of the supernatant:

(a) *Determination of NR_{act}*: 100 µL of *Pichia* extracts were mixed with either 100 µL of desalted darkened-leaf extracts or extraction buffer. The mixture was incubated for 15 min at 24°C and then added to 800 µL reaction buffer (100 mM HEPES, if not mentioned otherwise, pH 7.6, 5 mM DTT, 10 mM $MgCl_2$, 10 µM FAD, 10 µM sodium molybdate, 50 µM Leupeptin, 2 mM Pefabloc, 5 mM KNO_3 and 0.2 mM NADH). The reaction was carried out at 24°C. After 10 min the reaction was stopped by adding 125 µL zinc acetate (0.5 M).

(b) *Determination of NR_{max}*: The procedure was carried out as described above, except that the reaction buffer contained 10 mM EDTA instead of 10 mM $MgCl_2$.

Excess NADH was removed by 10 µM phenazine methosulphate (PMS) treatment. The colorimetric determination of formed nitrite was carried out as described previously (Hageman and Reed, 1980).

4.9.3 ATP-dependent Inactivation *in vitro*

Leaf extracts: Ricinus used in this experiment were 3-week old plants. All plants were illuminated at about 10 a. m. for one hour before harvesting the leaves. The composition of extraction and reaction buffers was described above but all were

adjusted to pH 7.3. 100 µL of the leaf extracts from Arabidopsis, spinach and Ricinus with or without 2 mM ATP plus 50 µM Cantharidin were preincubated for 15 min at 24°C. Subsequently, 900 µL of reaction buffer containing either 10 mM $MgCl_2$ or 10 mM EDTA was added to the incubation mixtures. After a 5-min incubation, the substrates (5 mM KNO_3 and 0.2 mM NADH) were added and the reaction was carried out for 5 min (3 min for spinach leaf extracts). Nitrite was assayed as before.

Mixtures of recombinant NR's and NR-free leaf extract: The desalted, NR-free extracts of Arabidopsis, Ricinus and spinach 4-day darkened leaves were used in the *in vitro* inactivation assay. 100 µL of *P. pastoris* extracts containing either Arabidopsis NR2 or Ricinus NR were mixed with 100 µL of plant leaf extracts or extraction buffer. The mixture was then incubated at 24°C for 15 min without or with ATP (2 mM) plus 50 µM Cantharidin in the presence of 10 mM $MgCl_2$ in order to inactivate NR. 800 µL of reaction buffer (10 mM Mg^{2+} or 10 mM EDTA) was then added to the mixtures and incubated for 5 min. Thereafter, 5 mM KNO_3 and 0.2 mM NADH were added and the reaction was carried out for 10 min and nitrite was determined as before.

4.10 Gas Phase NO Measurements

The principle for measuring nitrogen dioxide (NO_2) is the gas phase chemiluminescense reaction of nitric oxide (NO) with ozone (O_3). The reactions between NO and an excess amount of O_3 can be described by the following formulae:

$$NO + O_3 \rightarrow NO_2 + O_2 \quad [1]$$
$$NO + O_3 \rightarrow NO_2^* + O_2 \quad [2]$$
$$NO_2^* \rightarrow NO_2 + hv \quad [3]$$
$$NO_2^* + M \rightarrow NO_2 + M \quad [4]$$

NO_2^*: the excited nitrogen dioxide molecule
M: deactivating colliding partners (N_2, O_2, H_2O)

The spontaneous deactivation of NO_2 occurs with emission of light [3]. By far the larger fraction of NO_2^* loses its excitation energy without light emission by colliding with other molecules (M) [4]. In order to achieve a high yield of light the reaction of NO with O_3 needs to take place under low pressure. The light intensity generated from the chemiluminescent reactions [2] [3] is proportional to the mixing ratio of NO. A photomultiplier tube is used to convert the light energy emitted from [3] into electrical

impulses. The electrical impulses are counted over a chosen time interval. A microprocessor calculates the NO signal in ppb.

For experiments with *Pichia* transformants, 1 mL of the cells (48 h induction) were collected and resuspended in 1 mL of fresh BMG medium (see 4.7) in a 5 mL beaker. The cells were then placed in a plastic bottle with 200 mL air volume and were continuously stirred. A constant flow of measuring gas (purified air or nitrogen) of 1.5 L/min was pulled through the plastic bottle and subsequently through the chemiluminescence detector (CLD 770 AL ppt, Eco-Physics, Dürnten, Switzerland, detection limit 20 ppt; 1 min time resolution) by a vacuum pump connected to an ozone destroyer. The ozone generator of the chemiluminescence detector was supplied with dry oxygen (99%). The measuring gas (air or nitrogen) was made NO free by conducting it through a custom-made charcoal column (1 m long, 3 cm internal diameter, particle size 2 mm). After 5 min monitoring the baseline under the aerobic condition, 1 mM nitrite was added to the cell culture as the substrate for recombinant NRs. NO emission was monitored for 5 to 10 min and then the measuring gas was switched to nitrogen to provide anoxic conditions. After 5 to 10 min the sample was brought back to air. Calibration was carried out with NO free air (0 ppt NO) and with various concentrations of NO (1 to 35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen, Messer Griesheim, Darmstadt, Germany) with NO-free air. Flow controllers (FC-260, Tylan General, Eching, Germany) were used to adjust all gas flows.

4.11 List of Chemicals

Chemicals (Enzymes)	Company
6-Aminocapronic acid	Fulka, Neu-Ulm
10× TBS	Bio-Rad, München
Acetic acid	AppliChem, Darmstadt
Acrylamide/bisacrylamide	Roth, Karlsruhe
Agarose	ICN, Eschwege
Ammonium persulfate	Bio-Rad, München
Ammonium sulfate	Merck, Darmstadt
ATP	Sigma, Deisenhofen
Biotin	Sigma, Deisenhofen
Boric acid	Grüssing, Filsum
Bovine serum albumin (BSA)	Biomol, Hamburg
Cantharidin	Biomol, Hamburg
Casein	Sigma, Deisenhofen
DEPC	Roth, karlsruhe
Dextrose	Merck, Darmstadt
DTT	Biomol, Hamburg
EDTA	Merck, Darmstadt
Ethidium Bromide	Roth, Karlsruhe
FAD	Sigma, Deisenhofen
Glycerol	Sigma, Deisenhofen
HEPES	Gerbu, Gaiberg
Histidine	Sigma, Deisenhofen
Leupeptin	Biomol, Hamburg
Methanol	Roth, Karlsruhe
MOPS	Merck, Darmstadt
NADH	Biomol, Hamburg
NADPH	Biomol, Hamburg
Pefabloc	Biomol, Hamburg
Phenol	Roth, Karlsruhe
PMS	Sigma, Deisenhofen

p-nitro-blue tetrazolium chloride	Roth, Karlsruhe
Polyvinylpolypyrolidone (PVPP)	Sigma, Deisenhofen
Potassium acetate	Fulka, Neu-Ulm
Potassium nitrate	AppliChem, Darmstadt
Potassium phosphate	Merck, Darmstadt
Restriction enzymes	MBI, Heidelberg
Sephadex G-25	Sigma, Deisenhofen
SDS	Roth, Karlsruhe
Sodium acetate	Sigma, Deisenhofen
Sodium molybdate	Merck, Darmstadt
Sulfanilamide	Serva, Heidelberg
TEMED	Sigma, Deisenhofen
Tris	ICN, Eschwege
Triton X-100	ICN, Eschwege
Tryptone	AppliChem, Darmstadt
Tween 20	Fulka, Neu-Ulm
Yeast extract	ICN, Eschwege
Zinc acetate	Merck, Darmstadt

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6 Appendix

6.1 Abbreviations

%	percent
AICAR	5-aminoimidazole-4-carboxiamide riboside
Ala	alanine; A
Arg	arginine; R
Asp	aspartic acid; D
AtNR2	*Arabidopsis thaliana* NR2
ATP	adenosine 5' triphosphate
BMG	buffered minimal glycerol
BMM(H)	buffered minimal methanol (histidine)
bp	base pair
BSA	bovine
CaMV 35S	Cauliflower mosaic virus 35s promoter
cDNA	complementary DNA
cm^2	square centimeter
Cys	cysteine; C
d	day
DNA	deoxyribonucleic acid
dNTP	2'-desoxyribonucleosid-5'-triphosphate
DTT	1, 4-dithiothreitol
EDTA	ethylenediamine tetraacetate
FAD	flavine adenine nucleotide
FW	fresh weight
g	gram
Gln	glutamine; Q
Glu	glutamic acid; E
GOGAT	glutamate synthase
GS	glutamine synthetase
h	hour
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
His	histidine; H
kDa	kilo dalton
LB	Luria-Bertani
M	molar
mA	milli Ampere
min	minute
mL	milliliter
mM	millimolar
MOPS	4-morpholinic-propansulfonic acid
mRNA	messenger RNA
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NO	nitric oxide
NR	nitrate reductase
NRA	nitrate reductase activity
OD	optical density

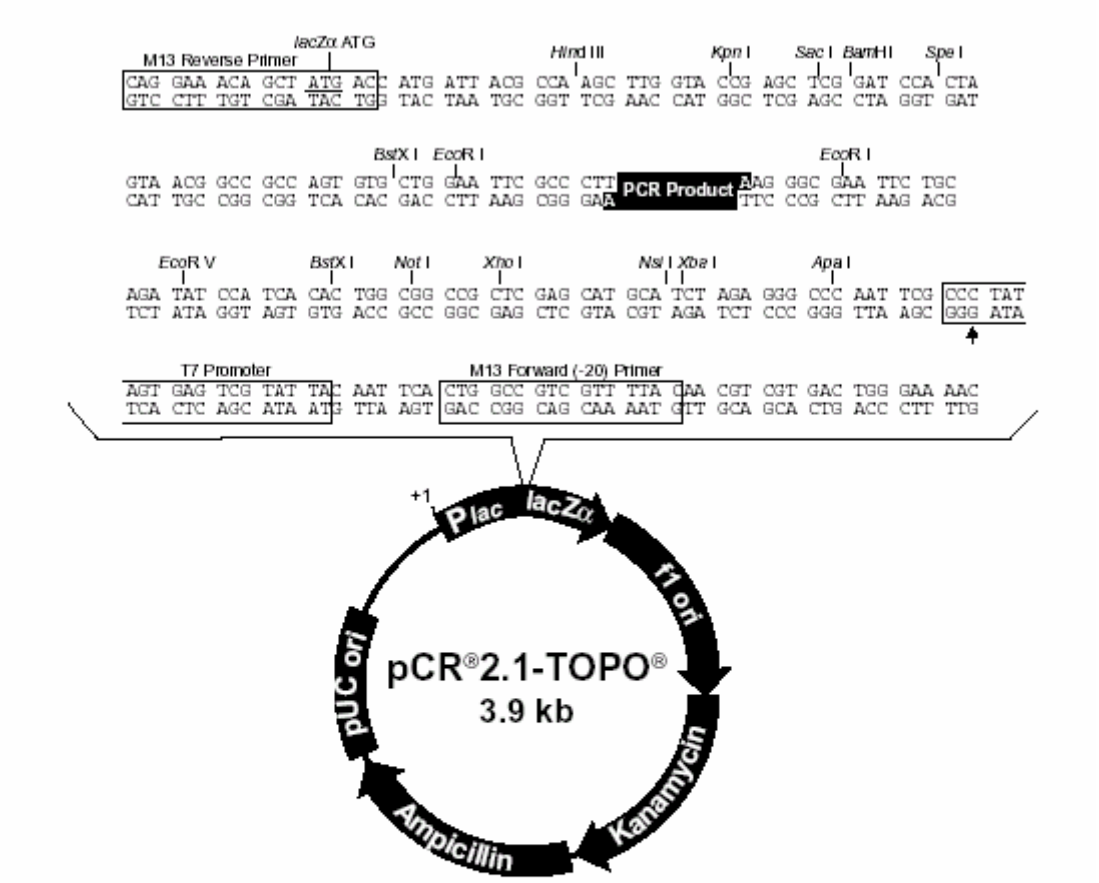
PAGE	polyacrylamide-gelelectrophoresis
PCR	polymerase chain reaction
pH	potential of Hydrogen, pH scale is logarithmic
phospho-NR	phosphorylated-nitrate reductase
PHYLIP	Phylogeny Inference Package
ppb	part per billion
Pro	proline;P
PVPP	polyvinylpolypyrrolidone
RACE	Rapid Amplification of cDNA Ends
RcNR	*Ricinus communis* NR
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulfate
Ser	serine; S
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
TEMED	N,N,N,N-tetramethylaminomethane
Thr	threonine; T
T_m	annealing temperature
Tris	Tris-(hydroxymethyl)-aminomethane
V	volt
v/v	volume/volume
Val	valine; V
w/v	weight/volume
UV	ultraviolet

6.2 Oligo Nucleotide Primers

Name	**Nucleotide Sequence**	**T_m**
RcNR-S1	5'-ATTGGTGGAAGAATGGTAAAGTGG -3'	59.3
AS-Arabidopsis	5'-CACGAACAATCTCTTTGGCAC-3'	62.7
RcNR-S-RACE	5'-GCTGAAGCATGGTGGTATAAGCCGG-3'	66.3
RcNR-AS-RACE	5'-TCTCTTTGGCACCAAGGAGGTCCAG-3'	66.3
5'-*AOX1*	5'-GACTGGTTCCAATTGACAAGC-3'	57.9
3'-*AOX1*	5'-GCAAATGGCATTCTGACATCC -3'	57.9
RcNR-SF-2	5'-AAACCGCGGAATAATGGCGGCCTCTGTGGACA ACAGGC-3'	>75
RcNR-ASF	5'-TAGAATACTAGCAATGAATTCTTGATATC-3'	59.9

6.3 Vectors

6.3.1 pCR® 2.1-TOPO

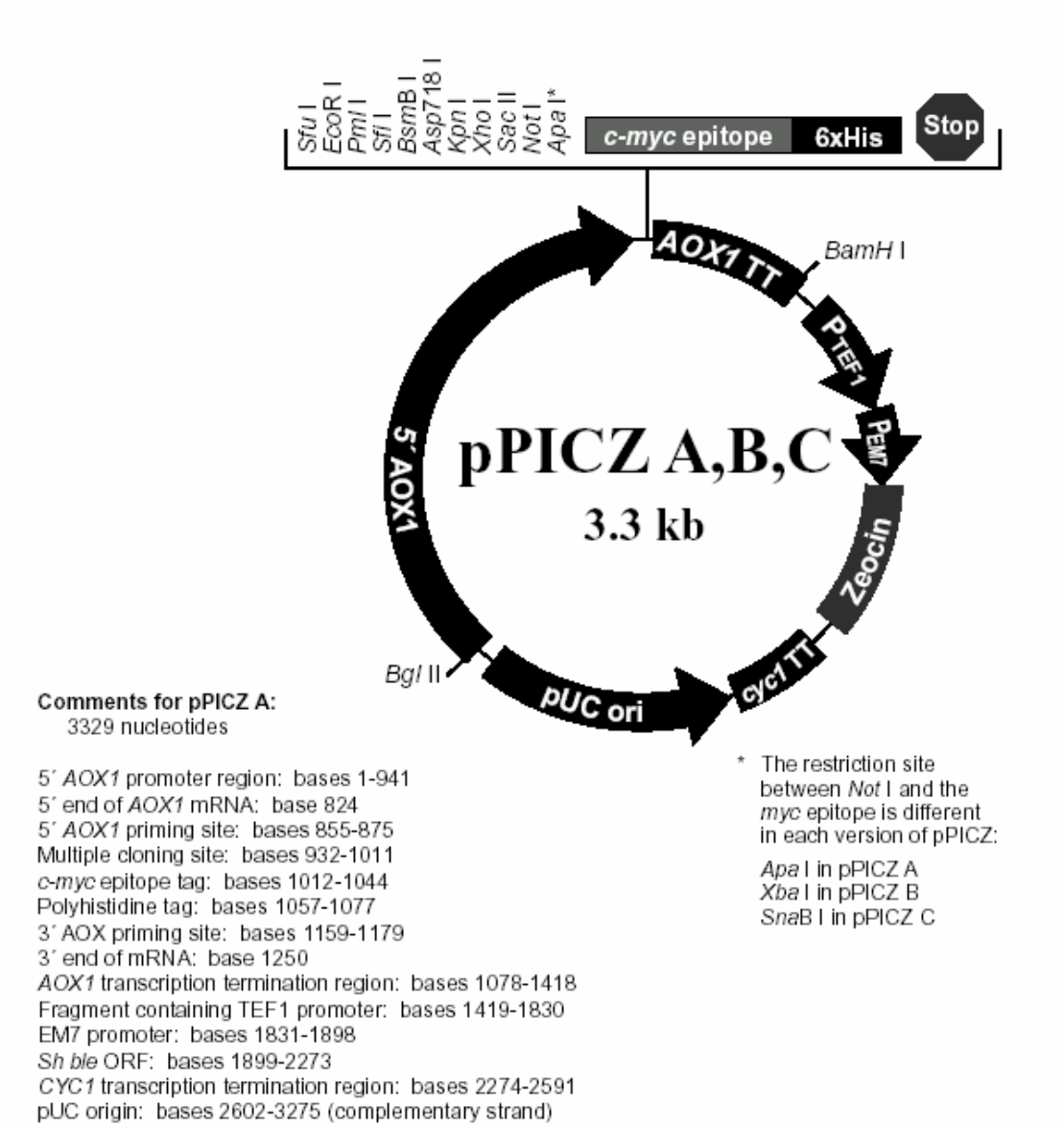


Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

source: http://www.invitrogen.com/content/sfs/vectors/pcr2_1topo_map.pdf

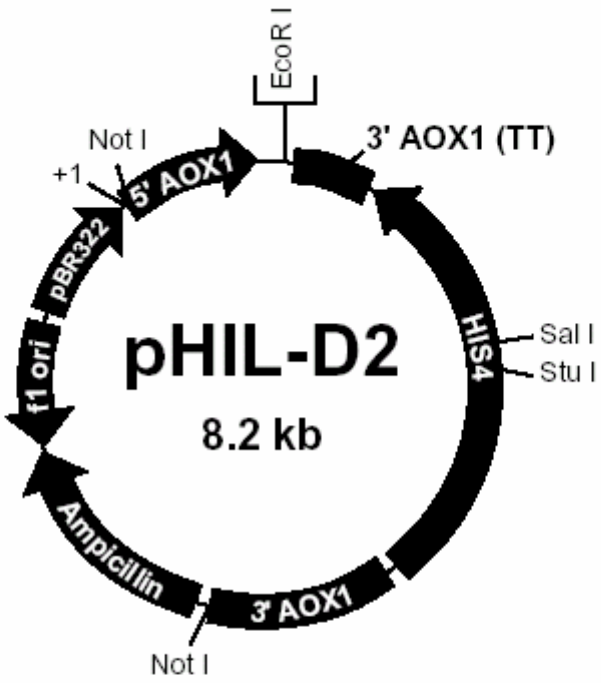
6.3.2 pPICZA



One *Nco* I restriction enzyme site: 1898; One *Sac* I site: 209

Source: http://www.invitrogen.com/content/sfs/vectors/ppicz_map.pdf

6.3.3 pHILD2



Comments for pHIL-D2:
8209 nucleotides

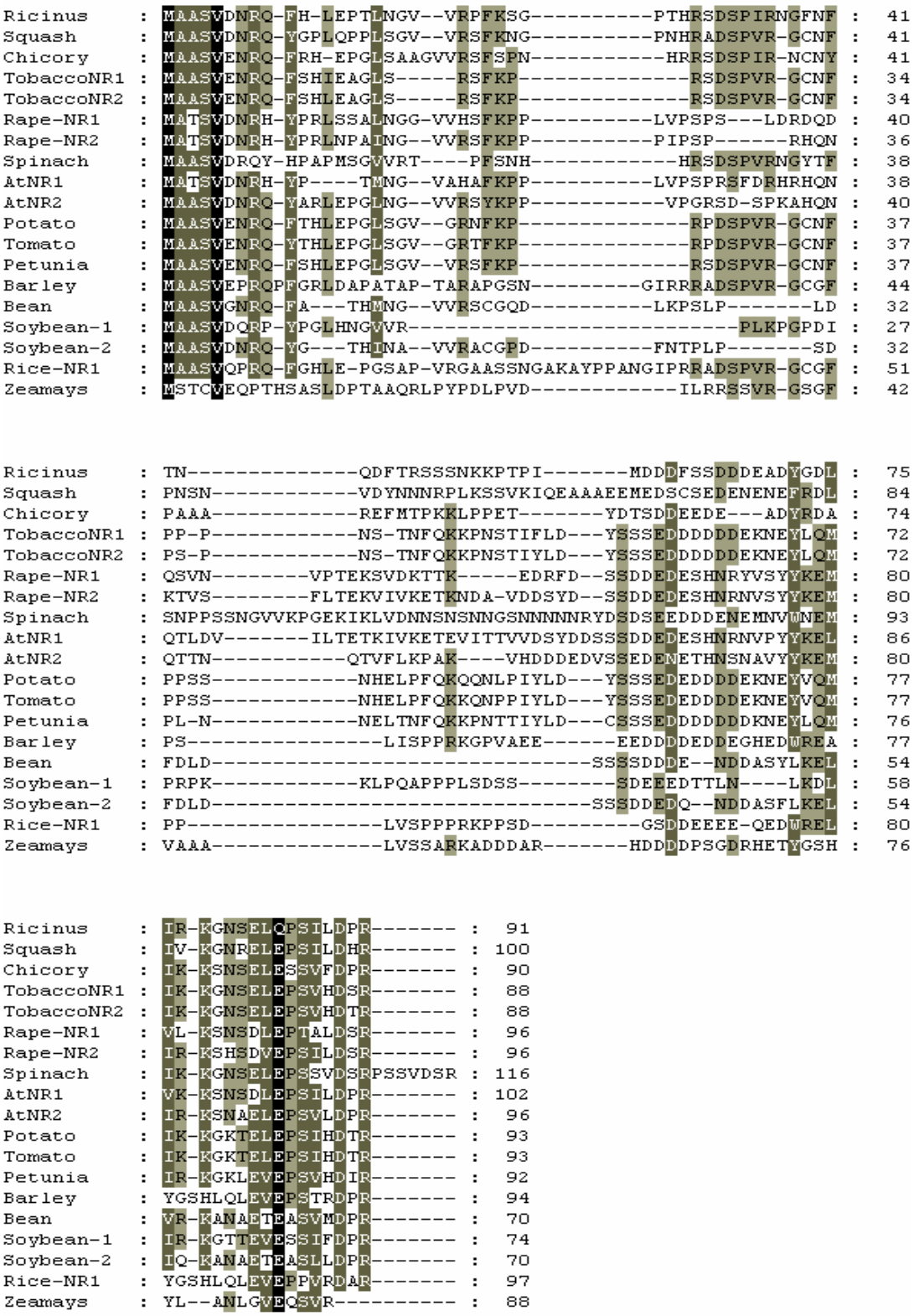
5' *AOX1* promoter fragment: bases 14-941
5' *AOX1* primer site: bases 868-888
*Eco*R I Site: bases 956-961
3' *AOX1* primer site: bases 1036-1056
3' *AOX1* transcription
termination (TT) fragment: bases 963-1295
HIS4 ORF: bases 4223-1689
3' *AOX1* fragment: bases 4578-5334
Ampicillin resistance gene: bases 5686-6546
f1 origin of replication: bases 7043-6588
pBR322 origin: bases 7138-7757

Source: http://www.invitrogen.com/content/sfs/vectors/phild2_map.pdf

6.4 Key invariant residues in Ricinus NR

Domain / Region	Key Residues	Function
Mo-MPT	*8*	*Nitrate reducing / Active site*
	Arg139	Nitrate binding
	His141	MPT binding
	Cys186	Mo ligand
	Arg191	Nitrate binding
	His286	MPT binding
	Arg291	MPT binding
	Gly300	Mo ligand
	Lys304	MPT binding
Dimer interface	*2*	*Formation of stable dimer*
	Glu352	Ionic bond at interface
	Lys391	Ionic bond at interface
Hinge 1	*1*	*Regulatroy*
	Ser526	Phosphorylated
Cytochrome b	*2*	*Heme-Fe binding*
	His569	Heme-Fe ligand
	His592	Heme-Fe ligand
FAD	*5*	*FAD binding / Active site*
	Arg709	Binds FAD
	Tyr711	Binds FAD
	Gly742	Binds FAD
	Ser745	Binds FAD
	Lys748	Binds NADH
NADH	*3*	*NADH binding / Actice site*
	Gly791	Binds NADH
	Cys886	Active site
	Phe914	C-terminal

6.5 Amino acid sequence alignments (N-terminus)



6.6 Amino acid sequence alignments (without N-terminus)

Three prosthetic domains are marked by a shadow. Identical sequences are indicated by a dot, and gaps introduced for alignment purpose by a dash. Unique amino acid residue of Ricinus NR is labeled in blue color. T. platyph: *Tilia platyphyllos*, N. sylvest: *Nicotiana sylvestris*, N. tabacum: *Nicotiana tabacum*.

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Ricinus     : DEGTADNWVERHPSMIRLTGKHPFNSEAPLTQLMHHGFITPVPLHYVRNHGPVPK : 146
AtNR2       : ..Y...S.I..N..............A...R.................H...  : 151
Spinach     : ......Q.I..N.................R.....................N  : 164
AtNR1       : ..S...S.I..NS...........A.A..PR.................A...  : 157
Lotus       : .Q......IS.NS...............PR.....................  : 132
Bean        : ......Q..A.NA...........A.S..QR.....................  : 125
Soybean     : ......Q.IP.NA....F.......G.G..PR.........S..R.......  : 125
Peach       : ..A.S.S..H.N............C.A..AR.................V...  : 137
Squash      : ........I..NA................R.................V...  : 155
Birch       : ..A.....I..NA..............A...R.........A.........  : 140
T. platyph  : ..A.....I..N..............A...R.....................  : 132
N. sylvest  : ........I..NF................R.....................  : 143
N. tabacum  : ........I..NF................R.....................  : 143
Petunia     : ........I..NN................AR.....................  : 147
Tomato      : ........I..NF................SR.....................  : 148
Potato      : ........I..NF................AR.....................  : 148
Brassica    : ..S.G.K.IH.NS...........A.A..PR.................G...  : 151
Maize       : ......A.I..N.C.........L.C....AR.........A.........A...  : 148

Ricinus     : ASWKDWTVEICGLVKKPTRFTMDQLVNDFPSRELPVTLVCAGNRRKEQNMVKQTI : 201
AtNR2       : .Q.AE.....T.F..R.M........S..AY..FAA................KSK  : 206
Spinach     : .K.E......T....R.I.........D.Q...F..............T..S.  : 219
AtNR1       : .N.S......T....R.A....E...S......F.....................  : 212
Lotus       : .R.D......T....T.......R..RD.........................S.  : 187
Bean        : .N.E......T....R.......R..R...H..F.A..............K..  : 180
Soybean     : IK.DE.....T....RS.H...EK..R...H..F.A...........H.....S.  : 180
Peach       : G..Q......T....R.A....E...R..K...F.....................  : 192
Squash      : .K.A......C....R.A...........RF..F.A..............S.  : 210
Birch       : .R.E......C....R.A.....R..T..R...F................K..  : 195
T. platyph  : ...D......T......M.L..V......Q...F................K..  : 187
N. sylvest  : G..D......T....R.M...........C.....................  : 198
N. tabacum  : G..D......T....R.M.....................................  : 198
Petunia     : GM.D......T....R.M....E.....................................  : 202
Tomato      : ...S......T....R.M..............F.....................  : 203
Potato      : ...A......T....R.M.....................................  : 203
Brassica    : .E.S......T....R.AGL..E...S......F.....................  : 206
Maize       : GD.AT.....T....R.A.L..E..ARD..AV.I....A...........Q...  : 203

Ricinus     : GFNWGAAGVSNSVWRGVPLHFVLKRCGIYSRKKGALNVCFEGAEDLPGGG---GS : 253
AtNR2       : .....S....T........CD.......F...G.........S......AGTA..  : 261
Spinach     : .....S.A..T........RD.......M.SL..................---..  : 271
AtNR1       : .....S....T........SE..........RG..................---..  : 264
Lotus       : .....S....T......S.RH.....R.Q..SR...H.....D.......---..  : 239
Bean        : .....S..T.T........RH.......L..G....H.S...........---..  : 232
Soybean     : ........G.T........RH.......LA.M...MY.S...........---..  : 232
Peach       : .....P....N........CD........G..S..................---..  : 244
Squash      : ..........T....R...CD.......L.....................---..  : 262
Birch       : ..........T........RD.......F..GR..F..............---..  : 247
T. platyph  : .S........T......L.CD.......F...HR.................---.P  : 239
N. sylvest  : .......A..T........RA.......F..N...........DV.....---..  : 250
N. tabacum  : .......A..T........RA....Y..F..N...........DV.....---..  : 250
Petunia     : .......A..T........RA..........T...........DV.....---..  : 254
Tomato      : .......A..T........RA.......Q.............SDV.....---..  : 255
Potato      : .......A..T........RP.......H.............SDV.....---..  : 255
Brassica    : .....S....T......A.SD..........RG..................---..  : 258
Maize       : ..........T.....AR.RD.......VP..G..................---..  : 255
```

```
Ricinus     : KYGTSIKKEYAMVPSRDIILAYMQNGELLAADHGFPVRMIIPGFIGGRMVKWLKR : 308
AtNR2       : ............D.............Y.TP.......................... : 316
Spinach     : .........F..D.A............K.SP.......................... : 326
AtNR1       : .........M..D.A.............TP.......................... : 319
Lotus       : ..S......V..D.............V..P.......................... : 294
Bean        : ......S..M..D.............P..P.......................... : 287
Soybean     : .........M..D.............P..P.......................... : 287
Peach       : .........V..D.............Q.MP.......................... : 299
Squash      : .........L..D.A............Q..P.......................... : 317
Birch       : .......Y.M..D.A.....G......R.SP.......................... : 302
T. platyph  : .........F..D.............R..P.......................... : 294
N. sylvest  : .........F..D.A............K..P.......................... : 305
N. tabacum  : .........F..D.A............K..P.......................... : 305
Petunia     : .........F..D.............K.TP.......................... : 309
Tomato      : .........F..D.............M.SP.......................... : 310
Potato      : .........F..D.............M.SP.....................P..  : 310
Brassica    : .........M..D.A.............TP.......................... : 313
Maize       : ......T..W..D.............P.LP............C............ : 310
```

```
Ricinus     : IIVTTKESDNYYHYKDNRVLPSHVDAELANAEAWWYKPEYIINELNINSVITTPS : 363
AtNR2       : ..........F...........L......DE.G.....................C : 371
Spinach     : .....T.......................S......Q.................C : 381
AtNR1       : ....PQ...S...........L.......S.......................G : 374
Lotus       : ....EE.C.GH..................E.G.....................C : 349
Bean        : ....EQ.CESH..............P....E.G.....................C : 342
Soybean     : ....EH.C.SH..................D.G.....................C : 342
Peach       : .....R.....................................................C : 354
Squash      : ........E.................D............H..............C : 372
Birch       : ..............N...........D..K.........H..............C : 357
T. platyph  : ........ES.....................................X....M.C : 349
N. sylvest  : .....Q...S...........P........T.....................C : 360
N. tabacum  : .....Q...S...........P........T.....................C : 360
Petunia     : .....Q..ES...........P.............................C : 364
Tomato      : .....Q..ES...........P.............................C : 365
Potato      : .....Q..ES...........P..EL.......................... : 365
Brassica    : ....PQ.................Y.....P.E.S.....................G : 368
Maize       : ....PA.....................................................G : 365
```

```
Ricinus     : HEEVLPINSWTTQRPYTLKGYAYSGGGKKVTRVEVTMDGGDTWQVCSLDHPEKPN : 418
AtNR2       : ........A.................................N..A...Q.....  : 426
Spinach     : ........A.............................D..D..E...Q..GS  : 436
AtNR1       : .A......A.............................D..S..E...Q.....  : 429
Lotus       : .D......A............S.....................F..A..QQ....  : 404
Bean        : .D..............V.........................H..T.........  : 397
Soybean     : ..............M..F.....................G.....T..C.....  : 397
Peach       : .....................................D.....T...Q.....  : 409
Squash      : ........A............S..............S.......T......A.  : 427
Birch       : ...................................K.R..A.........  : 412
T. platyph  : .D...........M..............X.......D.......T.........  : 404
N. sylvest  : ........A............S.....................ST.......T  : 415
N. tabacum  : ........A............S.....................T.......T  : 415
Petunia     : ..................S.....................N..T.........  : 419
Tomato      : ........A.................................S..T.......T  : 420
Potato      : ........A..............P..................S..T.......T  : 420
Brassica    : ........A.................................S..E...Q.....  : 423
Maize       : .D.......I....G...........................L..H.........  : 420
```

```
Ricinus    : KYGKYWCWCFWSLEVEVLDLLGAKEIAVRAWDETLNTQPEKLNWNVMGMMNNCWF : 473
AtNR2      : .....S.....I..... : 481
Spinach    : .....G.....I..... : 491
AtNR1      : .....D.....S..D.....F....D..I..... : 484
Lotus      : .....T.....A.....N.I..... : 459
Bean       : .....T.....G.....N.I..... : 452
Soybean    : .....A.....I..... : 452
Peach      : .....S..V.....Q.....I..... : 464
Squash     : .....S.....H.....I..... : 482
Birch      : .....AH.....I..... : 467
T. platyph : ...N.....S.....X..I..... : 459
N. sylvest : .....S.....I..... : 470
N. tabacum : .....S.....I..... : 470
Petunia    : .....S.....I..... : 474
Tomato     : .....S.....T.....I..... : 475
Potato     : .....R.....I...H.....I..... : 475
Brassica   : .....D.....S.....I..... : 478
Maize      : .....I..... : 475
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Ricinus    : RIKTNVCKRHKGEIGIVFEHPTVPANQSGGWMAKERHLEISSENHP-ILKKSVST : 527
AtNR2      : .....P.....L.G.E.....K.ADA..-S..... : 535
Spinach    : .....P.....Q.G.K.....R.....DSG.--T..RTA.. : 544
AtNR1      : .....P.....R.G.....Q.....SNN-T..... : 538
Lotus      : .....P.....Q.G..P.....K.....QQDSRP..... : 514
Bean       : .....P.....Q.G..P.....K...Q.Q-EAKPS..... : 506
Soybean    : .....P.....Q.G.....K...K..-ESNPT..... : 506
Peach      : .....Q.G.....K..D--..SNT-T..... : 516
Squash     : .....P.....Q.G.....DR.....T.SNQ-T..... : 536
Birch      : .....A.M.....A.....G.....R.KN..T..DANQ-S..... : 521
T. platyph : .....P.....L.G.....T.A....K.IDAN.-T..... : 513
N. sylvest : ...M....P.....Q.G.....A.A.Q-T..... : 524
N. tabacum : ...M....P.....Q.G.....A.A..-T..... : 524
Petunia    : .....P.....Q.G.L.....A.A..-T..... : 528
Tomato     : ...M....P.....Q.G.....AVA..-T..... : 529
Potato     : ...MKCV.P.....Q.G.....AVA..-T..... : 529
Brassica   : .....P.....R.G.....Q..K...SH.-T..... : 532
Maize      : ...V....P.....Q.G..P.....R.K...TAEAAA.-G..R.T.. : 529
```

```
Ricinus    : PFMN---TSSKTVSMAEVKKHNSADSCWIIVHGHVYDCTRFLKDHPGGTDSILIN : 579
AtNR2      : ....---.TA.MY..S.....C.....M.....S..... : 587
Spinach    : ....---.T..MY..S.....A.....N...A.....S..... : 596
AtNR1      : ....---.A..MY.IS..R.....A..... : 590
Lotus      : ....---.FT.MY.IS.....P..A.....AA.....: 566
Bean       : ....---.A..MF.VS.....S.P..A.....: 558
Soybean    : ....---.T..MYT.S..R...N...A.....W.....R.... : 558
Peach      : ....--T.....Y.LS..E....PQ.A....Q.....N.....A..... : 569
Squash     : ....---.A.N.YTLS.....PQ.A.....S..... : 588
Birch      : ....---....MF..S.....E.A.....H.....A..... : 573
T. platyph : ....---.D...F..S..R.....A.....N.....A..... : 565
N. sylvest : ....---.A..MY..S..R..S....A.....A..... : 576
N. tabacum : ....---.A..MY..S..R..S....A.....A.....S..... : 576
Petunia    : ....---.A..MY..S.....A.....A.....I..... : 580
Tomato     : ....---.A..MY..S..R....S..A.....A.....V..... : 581
Potato     : ....---.A..MY..S..R....S..A.....A.....V..... : 581
Brassica   : ....---.A..MY..S..R.....E.A.....S..... : 584
Maize      : ....TT-DVG.QFT.S..R..A.QE.A..A.....A..... : 583
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```
Ricinus     : AGTDCTEEFDAIHSDKAKKMLEDYRIGELVDS-TAYTTDSNASSPNNSVHGASNM : 633
AtNR2       : .........E...................IT--.G.SS..S--.........S.AV : 638
Spinach     : ............................IS--.G..S..SS--.G.....G.VY.: 647
AtNR1       : .........E...................IT--.G.DS-----...V.......F : 638
Lotus       : .........E...................IT--.G..S..S--.........N.EF : 617
Bean        : ............................IT--.G..SADS--.........N.EF : 609
Soybean     : .........E........Q..........TT--.C.NS..S--.S.P....R.DT : 609
Peach       : ..............................T--.T.AS..TSN...I....PHRT : 622
Squash      : ............................IT--.G.AS..SSN.....T.....N.: 641
Birch       : ............................IT--.G.VS..-----..S.......T : 622
T. platyph  : ............................IT--.G.AS..SMS...........M : 618
N. sylvest  : ............................IT--.G..S..-----.G.....S.SF : 625
N. tabacum  : ......................E......LT--.G..S..-----.G.....S.SF : 625
Petunia     : ............................IT--.G..S..S.---......S.SF.: 630
Tomato      : ............................IT--.G..S..S---..S....S.SI : 631
Potato      : ............................IT--.G..S..S---..T....S.SI : 631
Brassica    : .........E...................IT--.G.DS-----...V....G.SV : 632
Maize       : ..................A..DT......ITTG.G.SS.-------.....G.VL : 631
```

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Ricinus     : S-------QTPLAPIKEITPT-----PARNVALVP-------REKIACKLVKKES : 669
AtNR2       : FS--------L....G.A..-------V..L...NP------.A..PVQ..E.T. : 672
Spinach     : .G------LAG....T.AV.-------L.....N.-------.V..P....E.V. : 682
AtNR1       : GP--------L......L..-------QK.I...NP------....PV...E.T. : 672
Lotus       : KH---------.........TMSLPPL.R.K...I.-------....P....S.T. : 656
Bean        : IH---------....N...TI--PPL.P.S...N.-------.Q..P....S.T. : 646
Soybean     : IP---------.T....VITP------M.S...I.-------....P....S.T. : 642
Peach       : .SEDISFLVT.........-------.VKS...T.-------....P....A.T. : 663
Squash      : .---------H....R.AP-------VS.R...A.-------N...P....S.T. : 673
Birch       : .---------H.......A-------.L.....I.-------GA..PT...Y.K. : 654
T. platyph  : .---------F......DA-------.T.P.....-------....P....E.T. : 650
N. sylvest  : .--------.F......LV.------AQ.S...I.-------....P....D.Q. : 659
N. tabacum  : .--------.F......LV.------AQ.S...I.-------....P....D.Q. : 659
Petunia     : .--------GF......LA.------AV.S...I.-------....P....D.K. : 664
Tomato      : .--------.F......LVQ.-----.T.S...I.-------....P....D.Q. : 666
Potato      : .--------.F......LVQ.-----.T.S...I.-------....P....D.Q. : 666
Brassica    : MS--------L....RQLA.-------TK.I...NP------....PV...E.T. : 666
Maize       : .H---------....R.AVRA.---------..SNP------.D..H....G.KE : 662
```

```
Ricinus     : LSHDVRLFRFALPS-DDQVLGLPVGKHIFLCATIDEKLCMRAYTPTSTIDVVGYF : 723
AtNR2       : ......K......V-E.M.................D..................... : 726
Spinach     : ......R...G...-E.................N..D..................... : 736
AtNR1       : ......K........-E..Q.............N..D...........A..A..HI : 726
Lotus       : ......V........-E..Q.................G...........G..E.... : 710
Bean        : ..........E...-KN...................G.............EE..F. : 700
Soybean     : ..........G...-.GL....A........V..................HE.... : 696
Peach       : ..............-E...................EG..............E.... : 717
Squash      : ......V......GGQ..A.................G..............E..F. : 728
Birch       : .........L....-.....................D..............E...L : 708
T. platyph  : ..............-.................V...D..............E.DH. : 704
N. sylvest  : ......K........-E.................V..D..............E.... : 713
N. tabacum  : ..P...K........-E.................V..D..............E.... : 713
Petunia     : ......K........-E.................I..D..............E.... : 718
Tomato      : ......K........-E...................D..............E..F. : 720
Potato      : ......K........-E...................D..............E..F. : 720
Brassica    : ......R........-E..Q.................D..............A...I : 720
Maize       : ..R.......S...-P...................EG...........M..E..H. : 716
```

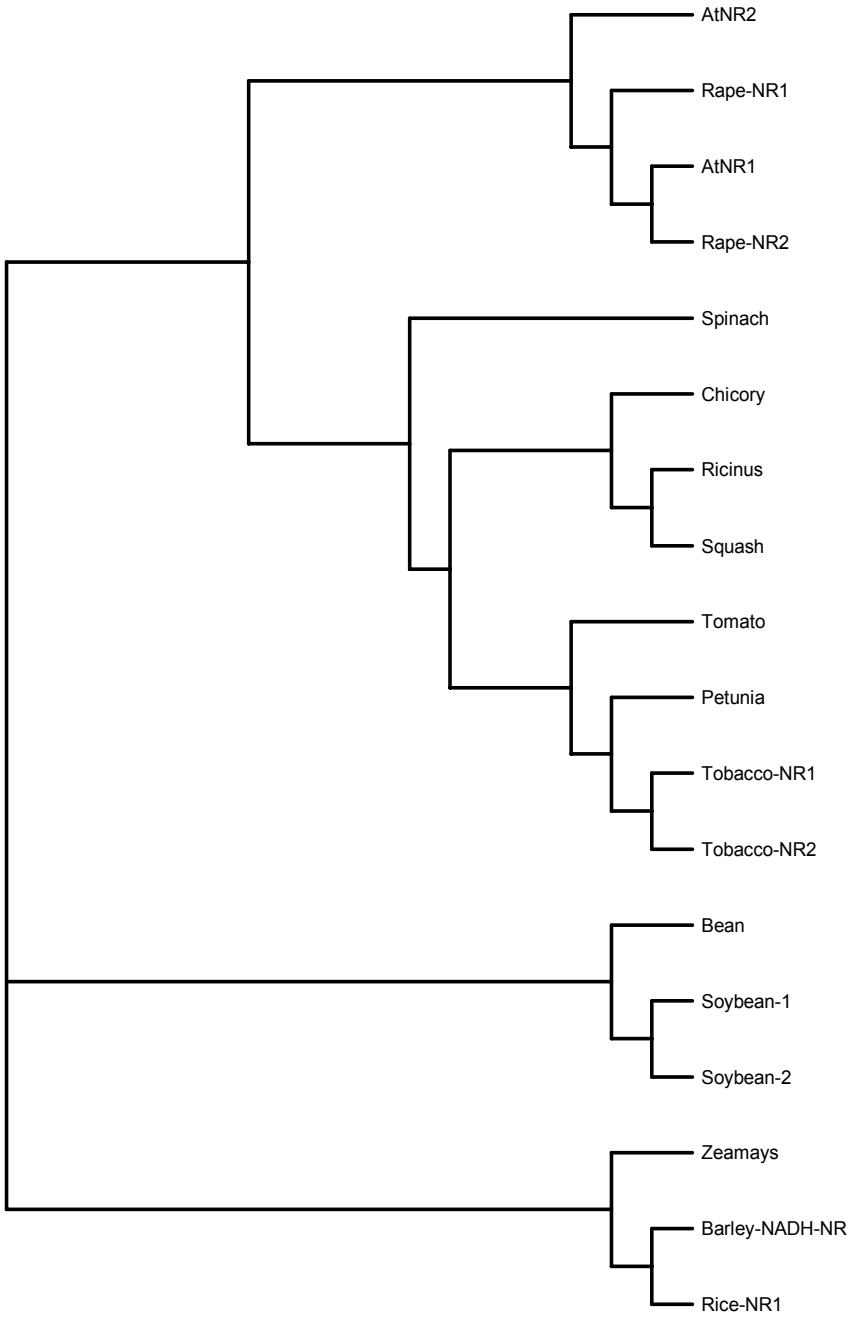
```
Ricinus     : DLVIKVYFKGVHPKFPNGGLMSQHLDSLQLG-SVIDVKGPLGHIEYAGRGNFMVH : 777
AtNR2       : E.......G.............Y....P..-.T.E.........L...S.T.. : 780
Spinach     : .........D.................S..-.I...........L.....T.. : 790
AtNR1       : .........D.................P..-.M...........K.....L.S : 780
Lotus       : E.................A........P..-.D...........T.....L.. : 764
Bean        : .........D.............Y.E..S..-.M...........T.....T.N : 754
Soybean     : ............................P..-.............T.....L.. : 750
Peach       : .........N.................P..-AA...........T...H.L.N : 771
Squash      : E.....................Y....E..-.T...........T.....M.. : 782
Birch       : .........NSN..............P..-...H.........T.....L.. : 762
T. platyph  : ......................Y....P..-.............T.....S.. : 758
N. sylvest  : E................Q...Y....P..-.F...........Q.....L.. : 767
N. tabacum  : E................Q...Y.......-.F...........Q.....L.. : 767
Petunia     : E..........V.......Q...Y....P..-AF...........Q.....L.. : 772
Tomato      : E................Q........P..-AF...........Q.....L.. : 774
Potato      : E................Q........P..-AF...........Q.....L.. : 774
Brassica    : .........N.............P...P..-A...........Q...K...S : 774
Maize       : .........NE............Y....P..-.Y...........T...S.V.N : 770
```

```
Ricinus     : GKPKFAKKLTMLAGGTGITPIYQVIQAILKD-PEDDTEMYVVYANRTEDDILLRE : 831
AtNR2       : ......D..A.....................-...E...........E...... : 834
Spinach     : .........A..S...........M......-...K...H........E...... : 844
AtNR1       : .........A..............S..S.-...E.................... : 834
Lotus       : ..H......A.............A......-...H.K................. : 818
Bean        : ..S......A.............A......-...L...H.............. : 808
Soybean     : ......T..A.............V......-...C...H.............. : 804
Peach       : .........A.............A......-...E...........V....... : 825
Squash      : .........A.............V......-...E...................D : 836
Birch       : .E.......A.....................-...E...F.............. : 816
T. platyph  : .........A.....................-...E................... : 812
N. sylvest  : ..Q......A.............M......-........................ : 821
N. tabacum  : ..Q......A.............M......-........................ : 821
Petunia     : ..R......A.............M......-...E...H.............D : 826
Tomato      : ..Q......A.............M.S....-.....................D : 828
Potato      : ..Q..P...A..............S....-........................ : 828
Brassica    : ......N..A..............S..S.-...E.................... : 828
Maize       : ..Q.H.S..A..C.....................H...H.............D : 825
```

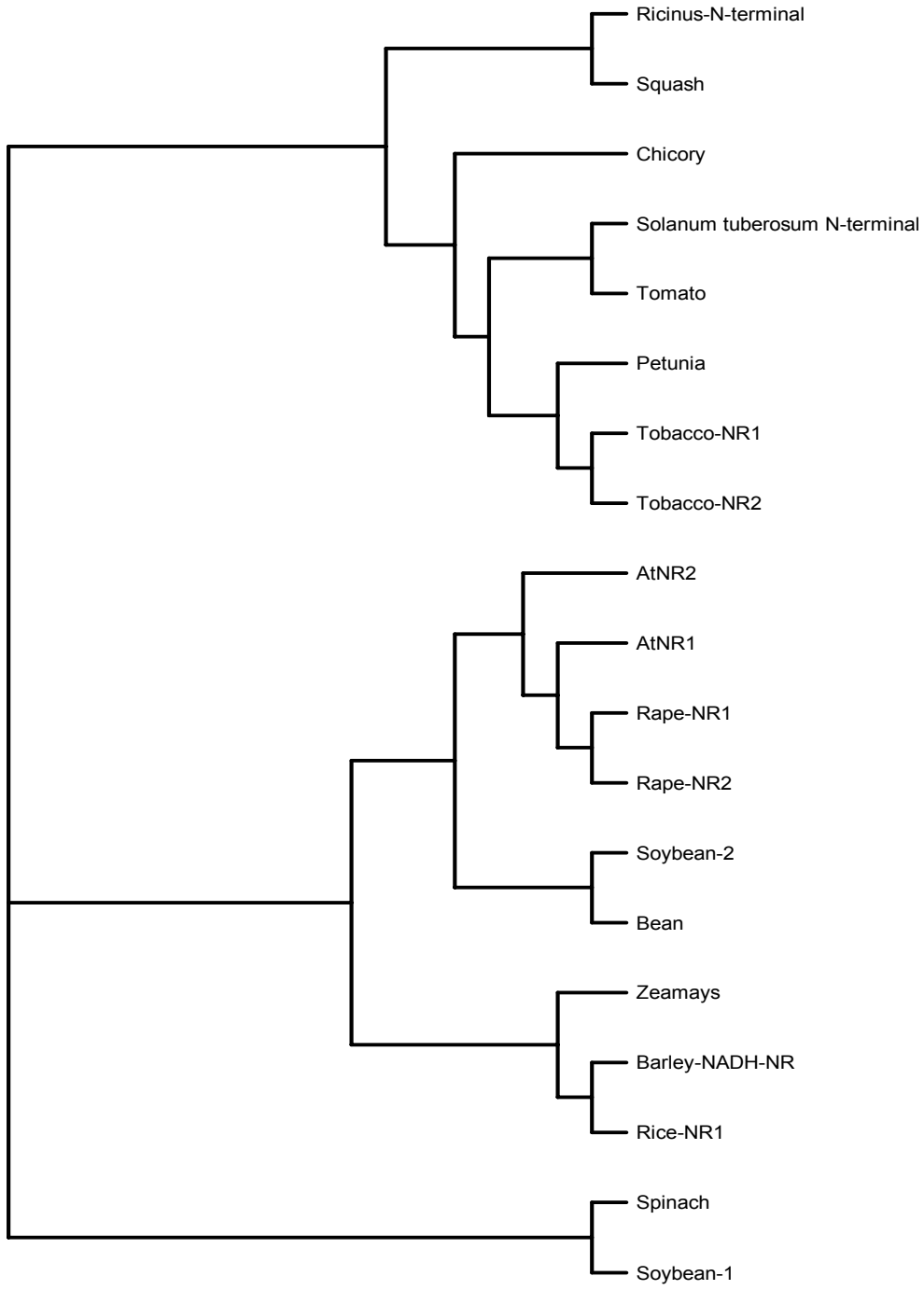
```
Ricinus     : ELDSWAKEHHERLKVWYVVQES--IKEGWQYSVGFITENILREHVPEGSD-DTLA : 883
AtNR2       : ...G..EQYPD.........ES---A....A..T.....A.......D.L.GSA.. : 886
Spinach     : ...K..D.FRD.........EK---AE...K.DT.....K...D...AVG.-.V.. : 895
AtNR1       : ..EG..SK.K..........EI---A....S..T.....A..........LEGES.. : 886
Lotus       : ...T...KYED.F.......ET---A....G.........G.........AG.-.A.. : 869
Bean        : ...T.....C..F.......ET---A....G.G......A.........A.S-.S.. : 859
Soybean     : ...E...KY-D............--.R...E........S..T....NA.P-.... : 855
Peach       : ...A...K-Y..F.......EN---GR...E.......DT.......D...-GS.. : 875
Squash      : ...T...K-NQ............--.R...E................AAAE-.... : 887
Birch       : ...D...K-..........K..--KR...E......R.S...........-.V.. : 867
T. platyph  : ...G...K-.D............--TRK..E..L.....R...D......K-.... : 863
N. sylvest  : ......EKIP..........D.--.....K..I.....A.........P.H-T... : 873
N. tabacum  : ......EKIP..........D.--.....K..L.....A.........P.H-T... : 873
Petunia     : ......VKLP..........D.--.....K..T.....A........LP.Q-T... : 878
Tomato      : ...A..EQVPN............--.T...K..T.....S.........P.H-T... : 880
Potato      : ...A..EQIPD............--.T...K..T.....A........QP.H-T... : 880
Brassica    : ..EG..SK.PD.........EI---A....E..T.....A..........LEGES.. : 880
Maize       : ...R..A..PD.........DQV..PE...K........A.........G.-.... : 879
```

```
Ricinus     : LACGPPPMIQFAVQPNLEKMNYDIKNSLLVF : 914
AtNR2       : ...................Q.N..EDF.I. : 917
Spinach     : .T..............D..G....EQ..I. : 926
AtNR1       : ...................G....ED..I. : 917
Lotus       : ............N......G.....D.... : 900
Bean        : .T..................G.....D.... : 890
Soybean     : .T..................G..TQ.N.... : 886
Peach       : .........K.......L.....TTD..... : 906
Squash      : ......A................T....... : 918
Birch       : ....A.S...E..RL........T....II. : 898
T. platyph  : ..............K.........D..... : 894
N. sylvest  : ............N......G....D..... : 904
N. tabacum  : ............N......G....D..... : 904
Petunia     : ............N......G....D..... : 909
Tomato      : ............N......G....EE.... : 911
Potato      : ............N......G....EE.... : 911
Brassica    : ...................G....ED..I. : 911
Maize       : ............S......K..MA..FV.. : 910
```

6.7 Phylogenies analysis (Full-length NRs)



6.8 Phylogenies analysis (N-terminus of NRs)



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1997-1999 Private study at Taichung City Buddhist Lotus Organization

1995-1997 Master of Science
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Research project: Quantitation and analysis of neuron growth inhibitory factor (Metallothionine III) gene transcripts
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Publication List

Tsai C-B, Kaiser WM and Kaldenhoff R (2003) Molecular cloning and characterization of nitrate reductase from *Ricinus communis* L. heterologously expressed in *Pichia pastoris*. Submitted for publication to Planta on 21.01.2003

Kaiser WM, Weiner H, Kandlbinder A, Tsai C-B, Rockel P, Sonoda M, and Planchet E (2002) Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. J. Exp. Bot 53: 875-882

Printed Documentation:

Tsai C-B, Kaldenhoff R, and Kaiser WM (2001) On the Way to Understand the Molecular Basis for Species-Specific Differences in the Regulatory Properties of Nitrate Reductase of Higher Plants. In: Tagungsdokumentation von DAAD-BioForum-Berlin. DOK&MAT 42: 119-121

Presentations:

DAAD-BioForum, Berlin 2001. Regulatory properties of nitrate reductase from *Ricinus communis* L.

Rhein-Main-Botaniker-Kolloquium, Heidelberg 2002. Special regulatory properties of nitrate reductase in *Ricinus communis* L.: heterologous expression of NR in *Pichia pastoris*.

Poster Presentation:

Fifth International Symposium, Nitrate Assimilation: Molecular and Genetic Aspects, NAMGA 2002, Córdoba, Spain. Regulatory Properties of Nitrate Reductase from *Ricinus communis* L.

ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation in allen Teilen selbst angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ich habe die Dissertation weder in gleicher noch in ähnlicher Form in anderen Prüfungsverfahren vorgelegt.

Ich erkläre weiterhin, dass ich bislang noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, Februar 2003

…….