NITRATE REDUCTASE STRUCTURE, ROLE AND FACTORS AFFECTING ITS REGULATION: A REVIEW

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Abstract

Nitrate Reductase is a key regulatory enzyme in the process of nitrogen assimilation that reduces the inorganic form of nitrogen that is nitrate into nitrite which finally assimilates into the various organic forms of nitrogen. NR is a dimeric protein comprising of two identical subunits and involves three prosthetic groups for catalysing the nitrate reduction process. NR utilizes physiological electron donor (ferredoxin) in a reduced form to facilitate the assimilation of nitrate. It is well known that the assimilation of nitrate is strongly regulated mostly by several endogenous and environmental factors in plants. The most noticeable of these is the availability of the source of nitrogen as an inducer (nitrate). Nitrate acts as an inducer for expression of mRNA transcript. Another factor, the light also induces NR mRNA transcription. Further, light/dark variations and many other factors like growth regulators, photosynthetic electron transport chain, environmental stress also play a crucial role in the NR regulation. This review explains the structure, role of NR in higher plants and the factors affecting its regulation.

Key words: Nitrate Reductase, Nitrate reductase activity, Heme domain, transcription and regulation.

Introduction

Inorganic nitrogen in the biosphere is converted by either the 'fixation' of molecular nitrogen (N2) or by 'assimilation' of nitrate into a biologically usable form of organic nitrogen. The number of bacteria, certain fungi and nearly most of the algae and crops possess the potential to assimilate nitrogen in the form of nitrate from soil (Hirel and Krapp, 2019). Major progress put in the direction of the integration of nitrogen-fixing genes has been done so far among higher plants. The conversion of the inorganic form of nitrogen into organic form is known as the nitrate assimilation process. Nitrate assimilation is expected to produce an organic form of nitrogen more than twenty thousand megatons in a year, compared to two hundred megatons for fixing the nitrogen with the help of microbes (Guerrero *et al*., 1981; Klein *et al*., 2000; Sharma and Dubey, 2005). Approximately, 25 percent of photosynthetic energy is used or nitrate assimilation. The method of assimilation of nitrate takes place by the reduction of nitrate into ammonia utilizing eight electrons. The use of eight electrons takes place in dual stages. In the first stage, a nitrite is formed by the reduction of nitrate using 2 electrons in the presence of the enzyme nitrate reductase (NR). In this reaction,

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NAD(P)H functions as the electron donor. In the second reaction, six electrons are required for the formation of ammonia from the reduction of nitrite in the presence of enzyme nitrite reductase. This step is associated with the PET (photosynthetic electron transport) chain in crops via a reduced form of ferredoxin (electron donor) that is the product of a light photosynthetic reaction. NR, which is the rate-limiting step and controls the process of nitrate assimilation, catalyzes the initial reaction (Beevers and Hageman, 1969). In plants and other nitrate-assimilating species, the enzyme is known to be a major bottleneck for growth, development and protein production. Therefore, to identify nitrate reductase properties related to its enzymatic effectiveness and its regulation have drawn the attention of many researchers for its extensive study.

In this review article, we have focussed on the structure, function and regulatory network of nitrate reductase (NR) in plants.

Structure of NR: localization, isoforms and subunits

The reduction of nitrate occurs in green tissue and plant roots. A large part of the reduction occurs in the leaves of most plants. During the early stages of growth, the root contribution to nitrate reduction tends to be particularly intensive (Oaks, 1979). Its location in the cell

is not well known. However, most of the reports are evidenced with the theory of its cytosolic presence in the plant cell. However, Kamachi *et al.*, (1987) through immuno-gold labeling techniques proved NR presence in the chloroplast of spinach and cytosol of spinach and the presence of NR in the cytosol was proved by Vaughn and Campbell, (1988) in an experiment on maize leaves.

In eukaryotes, three different isoforms of NRs have been known: (a) A NADH-specific NR which is found in the majority of plants, algae, etc (b) A NAD(P)H bispecific is found in the Senegal coral tree and white birch species (Friemann *et al.*, 1991; Stewart and Orebamjo, 1979) and (c) NADPH specific present only in fungi and did not found in any plant (Guerrero *et al.*, 1981). Only one type of NR isoform: NADH-specific is found in tomatoes and tobacco. There is both an NADH-specific as well as a NAD(P)H-bispecific isoforms are present in the monocots like rice, maize and barley (Kleinhofs *et al.*, 1988; Redinbaugh and Campbell, 1981). The NADH-specific isoform is found both in leaf and root tissues of barley and maize and implies most of the overall activity of the enzyme NR, while the NAD(P)H-bispecific isoform is reported mostly in root tissue. Due to mutations in NAD(P)H bispecific isoform in plant barley, the activity of the enzyme is either induced or derepressed (Warner *et al.*, 1987). In soybean, all three isoforms or NR is present. Out of three NR, the NADH specific is dominant and is found in most of the higher plant species. Some plants possess a single gene-encoded NR type only while others require more than two NR isoforms with a changed electron donor. The different number of genes encodes the three different enzymes for example in Arabidopsis and beans two genes encode for NR whereas, in rice, three genes are responsible (Caboche and Rouzé, 1990; Rohilla and Yadav, 2019). The nitrate reductase in tomatoes is coded by a single gene and different nitrate reductase genes present in the tobacco (Daniel-Vedele *et al.*, 1989; Vaucheret, Vincentz *et al.*, 1989).

The different biochemical properties of NR were extensively studied since the nineties. It's a dimeric
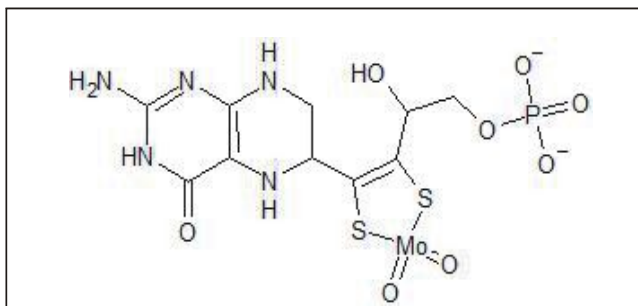

Fig. 1: Structure of molybdenum cofactor.

polypeptide of two identical subunits of 100 and 120 kDa. Three prosthetic groups are present in both of the subunits: (a) FAD- at C-terminal of the polypeptide, (b) heme or Cytb$_{557}$- found at the center and (c) MoCo center- in the N terminal region of the enzyme (Kramer, Johnson, Ribeiro, Millington and Rajagopalan, 1987) (Fig. 1).

Each domain contains redox centers. Protease and trypsin treatment in *staphylococcus* sp reveals the reason behind the dimeric structure of NR which is the 75 kDa Mo domain. It is reported that the FAD domain comprises 28 kDa whereas heme group is 14 kDa. All three domains of NR are interconnected with the help of hinge proteins (Fig. 2). Sometimes the NR had also been reported in tetrameric active form e.g. *Chlorella vulgaris*.

Electron transportation

The flow of electrons is from NAD(P)H electron donor to nitrate through FAD and Mo domains of NR:

$$NAD(P)H \longrightarrow FAD\ Cytb_{557} \longrightarrow Mo \longrightarrow NO_3^-$$

NR comprises 2 distinct active sites: including one NAD(P)H electron donation site, where FAD is reduced and the other one for the molybdenum cofactor where nitrate reduction occurs. NR acts like an electron transport network with electron transference starts from reduced FAD to the Mo cofactor. Besides nitrate reduction which is the main function of NAD(P)H, it also catalyzes some *in vitro* NR activities too e.g. NADH dehydrogenase. Its nitrate reductase activities further require few artificial electron donors *viz.* Flavin nucleotides, methylviologen and bromophenol blue.

Molecular structure

The entire NR nucleotide sequence has been obtained from many algae, plants and fungi. The identity varies from 63 to 91 percent of the peptide sequence of various plant NRs. Polypeptide sequence of most of the plants showed the forty percent similarity with the fungi Aspergillus and Neurospora species. The sequence similarities between algae and other higher plants found approx. to be 46 percent. In the case of rice, this similarity is about 69% with Arabidopsis and 67% with tobacco (Hemalatha, 2002). The number of introns present in different isoforms of NR also varies in different plants. For example, NADH and NAD(P)H specific isoforms of NR in barley and Arabidopsis consists of one to three introns respectively. The size of these introns varies in different plants but its position is evolutionary conserved *i.e.* present in the Mo domain of polypeptide. In the case of rice, three introns are present each of which is 85 bp, 108bp and 1954 bp long. In other monocots, use of codon biassing indicated that the codons plays a significant role in regulating the expression of the nitrate assimilating

genes (Choi *et al.*, 1989; Crawford *et al.*, 1988; Gao *et al.*, 2019; Hamat *et al.*, 1989; Lahners *et al.*, 1988).

The numbers of reports explaining the details of the NR structure have been provided employing the cloning and sequencing of plant NRs. It was possible to assign a functional domain to 3 protein regions relative to those of other proteins linking the same prosthetic groups in the NR sequences (Calza *et al.*, 1987; Crawford *et al.*, 1988; Hoff *et al.*, 1992). Additionally, another feature of NR is that it's a flavoenzyme which is a complex of heterogeneous proteins with prosthetic groups being FAD or FMN. NR shares a 47 percent identity with the cytochrome b5 reductase FAD domain, but no substantial identity with a number of other flavoenzymes can be identified. In the binding of NADH to cyt b5 component of NR two amino acids i.e. cysteine and lysine have a significant role. All plants have an effective expression of the NR holo-enzyme that is active. The tobacco nia2 gene was expressed in the NR deficient mutants of *Nicotiana plumbaginifolia* under both its own promoter and a constitutive promoter (Vaucheret *et al.*, 1990; Vincentz and Caboche, 1991). In different expression vectors, studies of cloned NR sequences would provide essential information on the detailed structure and role of NR. It is possible to perform site-directed mutagenesis of the NR protein and the outcomes can be evaluated using different kinds of expression vectors. The proper expression of NR and its crystallization provides detailed structural studies.

Regulation of NR

(a) Substrate induction:

In most plants nitrate acts as an inducer for the proper functioning of nitrate reductase. This means that, in response to nitrate, the activity, quantity and mRNA transcript accumulation of nitrate reductase enhances. It appears that the impact of nitrate is at the transcription level. An initial lag step followed by an exponential increase step and a steady-state level. This explains the kinetics of the nitrate induction process for the activity of NR. According to a hypothesis, during nitrate induction, de novo synthesis of NR is responsible for the increased NR activity (Deng *et al.*, 1990; Galangau *et al.*, 1988; Kuo *et al.*, 1981; Remmler and Campbell, 1986; Somers *et al.*, 1983; Yun *et al.*, 2008). Also, the activity of NR is lagged to the synthesis of NR protein. The explanation for this lag may be the time taken for the synthesis of the whole protein and the synthesis of an active enzyme from different cofactors. In several plant species including rice, the accumulation of NR mRNA due to nitrate induction has been reported. These studies showed that in the absence of nitrate there was no significant or very negligible level of mRNA in the roots and leaves. The addition of nitrate leads to a sharp rise in the NR mRNA level although it depends on the plant, tissue types and the timeframe. NR mRNA induction is significantly faster as compared to NR polypeptide synthesis and NR activity. NR activity is increased with a reduction in the level of mRNA transcript. RNA analysis and transcription assays with barley and soybean isolated nuclei, respectively, suggested that NR mRNA transcript increases due to synthesis of transcript and not due to pre-mRNA activation or decreased mRNA degradation. After peak accumulation upon nitrate induction, the decrease in NR mRNA level may be due to decreased mRNA synthesis or increased turnover. Compared to wild-type plants, some NR mutated plants including non-functional NR enzyme and molybdenum cofactor lacking mutants in *Nicotiana plumbaginifolia* display, mRNA over-expression when nitrate is supplied to them (Pouteau *et al.*, 1989). NR mRNA overexpression might have been the result of a significant decrease in nitrate-derived metabolites that usually regulate the NR gene expression level. In general, nitrate-only and ammonia-mixed nitrate treated plants synthesize enzymes at similar rates (Crawford *et al.*, 1988; Oaks *et al.*, 1988; Remmler and Campbell, 1986).
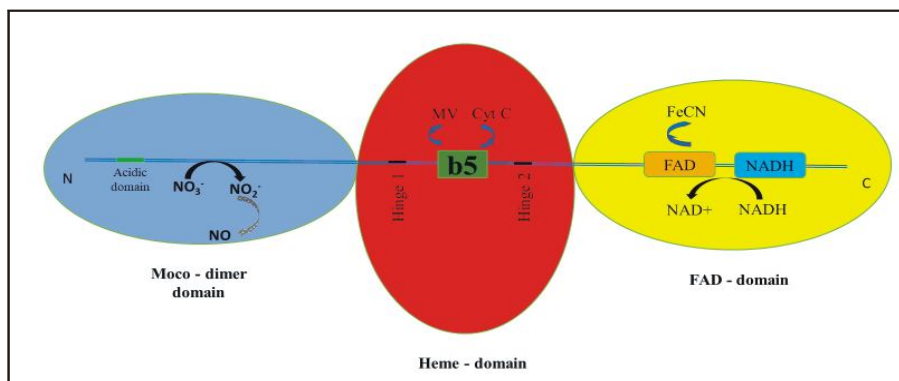

Fig. 2: Schematic shows NR domains. Different domains are presented in different colors (red: heme domain, blue: Mo domain, yellow: FAD domain). Crucial regulatory sequences are highlighted and present in hinge 1 and N-terminal peptides. Artificial electron (AE) donors are shown above while AE acceptors below the natural-physiological electron sources. Abbreviations: FeCN, potassium ferricyanide; NADH, NADH-binding motif; Moco, Moco-binding motif; dimer, dimerization region; FAD, FAD-binding region; Cyt.c, cytochrome c; b5, heme domain; MV, methyl viologen.

According to a report, Glutamine in the roots of soybean, tobacco and squash regulates NR by reducing the NR activity and NR transcription. Glutamine also reduced the concentrations of nitrates in squash plant cells. Glutamine can thus influence the regulation of NR through two main mechanisms. First, at the level of transcription, glutamine can participate in NR regulation. Secondly, glutamine might minimize nitrate levels by inhibiting the transportation of nitrate from extracellular space or vacuoles to the cytoplasm.

(b) Light induction:

Light has a powerful effect on NR polypeptide expression, activity and the level of mRNA. The light produces its impact on the expression of NR utilizing nitrate as a substrate. The mRNA transcript level and activity of NR induced at a low level in the presence of only nitrate substrate without the presence of light (Gowri and Campbell, 1989; Rajasekhar *et al*., 1988; Somers *et al*., 1983) but when the plants are again allowed for exposure to visible light, it strongly promotes the NR activity, mRNA levels and protein formation. The mutant tobacco plant for the NR gene was unable to synthesize NR. In the transformed plant with cDNA of the NR gene, the transcript was formed constitutively under the known promoter, but still for an accumulation of NR protein the light was needed. These findings showed that, apart from regulating NR transcription, the light also controls NR mRNA translation or NR protein permanency. The light-induced phytochromes as studied from the etiolated seedlings experiment. Among all the visible range, red and blue light have a stimulating or inducing role in increasing the activity and concentration of NR polypeptide. It also increases the NR transcript number during nitrate induction in many seedlings. However, barley seedling showed a negligible expression of NR suggesting that in green plants, phytochrome might not regulate NR expression in dark (Melzer *et al*., 1989).

(c) Diurnal differences in the NR expression:

NR activity and its mRNA transcript expression showed a diurnal pattern in Arabidopsis, tobacco, maize and tomato cultivated in the cycle of light and dark conditions in both leaves and root tissues under the influence of nitrate as substrate (Bowsher *et al*., 1991; Cheng *et al*., 1991; Deng *et al*., 1990; Galangau *et al*., 1988; Rohilla and Yadav, 2020). In tobacco and tomatoes, the amount of NR mRNA decreases steadily over time during the day and rises during the dark period. The level of mRNA was reported at the peak at the end of dark period. There was a fluctuation in the level of NR concentration for up to three to five hours which reflects the changes in the level of mRNA transcript, but with reduced intensity. NR activity increased at the highest level just after exposure to light and then declines as the light period is over. During the light cycle, the concentration of NR polypeptide and the level of its activity declines by approx. a factor of two, while mRNA accumulation decreases by 100-fold. This shows that the effect of different factors was more on the NR transcript level rather than on protein synthesis/degradation. Compared to the apoenzyme level, the fast increase in NR activity when light is turned on is due to the photoactivation of previously synthesized enzymes (Deng *et al*., 1990; Remmler and Campbell, 1986). In the NR mutants, diurnal fluctuations of NR mRNA synthesis did not take place either due to the effect on the NR gene or the effect on molybdenum cofactor encoding genes. In plants lacking a functional NR enzyme, the abolition of the diurnal NR mRNA transcript expression showed the involvement of metabolites that are derived from nitrate, such as glutamine role in the regulation of NR gene expression (Pouteau *et al*., 1989). NR mRNA transcription occurs continuously in the light and dark cycle in an NR gene mutant of *Nicotiana plumbaginifolia* which is transformed with the cDNA under the influence of a constitutive promoter. This suggests the role of diurnal oscillations in the mRNA transcript expression to regulate the nitrate reductase.

Shreds of evidence are present regarding the toxic nature of nitrate and nitrate reductase which reduces the nitrate present in the tissues uses a photosynthetic product i.e. reduced ferredoxin as an electron donor that might be connected to the regulation of NR activity by light. The stimulation effect of light on NR behavioral patterns may be mediated by a photosynthetic substance (Hoff *et al*., 1992; Solomonson and Barber, 1990; Yun *et al*., 2008).

(d) Other factors regulating NR:

The plastids played a significant role in the regulation of NR activity at the level of transcription in the presence of light and nitrate. A signal molecule originating from the plastids could perhaps mediate the plastidic regulation. It is also likely that the cell does have a system that controls the concentration of cytoplasmic nitrate and avoids nitrate accumulation by repressing the NR gene expression and then the need for intact plastids represents the inability of nitrite detoxification by damaged chloroplasts.

Also, fully developed photosynthetic machinery is required for the proper functioning or increased activity of NR. The researchers have observed the same enhanced NR activity and mRNA transcript expression

in green leaf tissues as compared to the etiolated plants (Gao *et al*., 2019; Gowri and Campbell, 1989; Rajasekhar *et al*., 1988; Rohilla and Yadav, 2019). This means that the photosynthetic machinery plays a significant role in the NR regulation.

Further, Kaiser and Huber, (1997), observed in higher plants that the regulation of NR involves the transcriptional or translational participating enzymes involved in the synchronization of nitrate reduction and carbon metabolism (Kaiser and Huber, 1997). Not only nitrate strongly affects the expression of NR genes at the transcription level, but also other factors *viz*. light, plant hormones, etc regulates NR gene expression (Lillo, 1994; Lillo *et al*., 2004; Solomonson and Barber, 1990). The NR enzyme protein is very short-lived, with a half-time degradation of a couple of hours. This elevated turnover rate enables nitrate reduction regulation depending upon the concentration of nitrate. However, a lot of situations have been reported over the years in which the rate of producible NRA does not match with the NR protein or the intensity of nitrates *in vivo* reduction. This suggests that there may still be certain regulatory mechanisms that regulate the protein's catalytic activity. It has also been reported that, in the NR regulation, NR protein synthesis or degradation was possibly not involved in the regulation of NR. The leaves of several other plants, including *Pisum sativum*, *Zea mays, Nicotiana tabacum* and *Arabidopsis thaliana* were also observed in the same way (Kaiser and Huber, 1997; Lea *et al*., 2006).

Furthermore, evidence for multisite seryl phosphorylation in Arabidopsis NR has been obtained *in vivo* suggesting the role of phosphorylation and dephosphorylation of NR enzyme in its regulation (La Brie and Crawford, 1994; Lea *et al*., 2006). Although the majority of higher plants studied so far has been extremely limited, thus it is not evidenced that phosphorylation of NR protein is responsible for the regulation of the polypeptide.

Besides all the above-mentioned factors influencing the regulation of nitrate reductase, environmental stress like water deficit conditions, salinity also affects the regulation of NR. Reduction in the water potential to less than -4 bar reported for reduced NR activity. This reduction in NR activity could be attributed to the fact that water stress might inhibit either protein synthesis or promotes its degradation process. Also, significantly lower transpirational pull while water stress might be responsible for a reduced influx of nitrate in plant tissues (Bardzik *et al*., 1971; Hirel and Krapp, 2019; Hsiao, 1970; Klein *et al*., 2000; Morilla *et al*., 1973; Plaut, 1973; Yun *et al*., 2008). The salinity effect on NR has not been studied so far. But in our previous study, it was reported that the promoter of NR genes gets influenced due to salinity presence and could be responsible for the reduced NR activity. The nucleotide sequences of the promoter region including the number of cis-regulatory elements differ in the different salt-responsive cultivars that might be the reason for the differential behavior of NR enzyme in terms of its activity (Rohilla and Yadav, 2019; Rohilla and Yadav, 2020).

Conclusions

The impact of different genetic and environmental factors influencing NRA in different systems is well known. But only a few studies explained the exact mechanism involved at the molecular level. However, it is evidenced that the enzyme is vulnerable to different parameters. The different factors can affect the enzyme's synthesis and/or activity either directly or by altering the cell organelles' physicochemical environment, by changing its transcript expression, inhibiting the synthesis/ degradation of NR protein by the process of phosphorylation/dephosphorylation. In several higher plant systems, the presence of an NR-specific inhibitor and NR vulnerability to different factors suggests the role of cis-regulatory elements, nitrous oxide, different transcription factors, secondary messengers and etc involved in the NR regulation and the enzyme activity. The information present in this review article might help to understand the various reasons responsible for the reduced NR activity which further could be used to enhance the enzyme responsible for sustainable agriculture.

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