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The role of non-symbiotic haemoglobin and nitric oxide homeostasis in waterlogging tolerance in *Vigna* species

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Abstract

The objective of this study was to examine the role of nitrate reductase, nitric oxide and non-symbiotic hemoglobin in imparting waterlogging tolerance in mung bean genotypes. Experiment was conducted with two cultivated mung bean [*Vigna radiata* (L.) Wilczek] genotypes T 44 (tolerant) and Pusa Baisakhi (susceptible) and a highly tolerant wild species *Vigna luteola* (Jacq.) Benth. The content of nitric oxide increased up to 6 d of waterlogging in *Vigna luteola* and T 44, and up to 4 d of treatment in Pusa Baisakhi. Increase in nitrate reductase (NR) activity was observed only up to 4 d of waterlogging in *Vigna luteola* and T 44, and up to 2 d of treatment in Pusa Baisakhi, and thereafter the activity decreased in all the genotypes. The increase in NO content and NR activity was greater in *Vigna luteola* and T 44 than in Pusa Baisakhi. *Non-symbiotic hemoglobin* (*NSHb*) and *cNR* mRNA expressions were observed only in waterlogging treated roots of *Vigna luteola* and T 44, while very little expression was observed in control plants of *Vigna luteola* and T 44, and in control and waterlogged plants of Pusa Baisakhi. PCR bands of *Hb* and *cNR* were cloned, and nucleotide and deduced amino acid sequences were obtained and conserved regions and domains were identified using database.

Additional key words: gene expression, mung bean, nitrate reductase, *Vigna luteola*, *Vigna radiata*.

Introduction

Waterlogging results in complete blockage of air (O_2) supply to the roots by clogging the soil air spaces, leading to hypoxic and subsequently anoxic conditions, and thus inhibiting root respiration. Under limited O_2 supply mitochondrial electron transport chain becomes inhibited and the intracellular content of ATP decreases as pyruvate fermentation yields only 2 mol of ATP per mol of glucose.

One of the major problems faced by plants/tissues experiencing hypoxia/anoxia is an increase in NAD(P)H/NAD(P) ratio, which adversely affects the glycolytic pathway, the only pathway which provides energy under anaerobiosis. To ameliorate this situation, plants use fermentative pathway employing alcohol dehydrogenase and lactic dehydrogenase for recycling NAD(P)H to NAD(P). Another important metabolic process, which may recycle NAD(P)H to NAD(P) is hypoxia induced nitrate reductase leading to formation of nitric oxide (NO). In this context role of non symbiotic hemoglobin and NO has also been highlighted in many plant species (Anderson *et al.* 1996).

Constitutive expression of barley class 1 non-symbiotic hemoglobin in transformed maize lines maintained cell adenine nucleotide contents and energy charge under hypoxic conditions (Sowa *et al.* 1998). Transformed alfalfa root cultures constitutively expressing barley hemoglobin maintained root growth during hypoxic treatment (Dordas *et al*. 2004). NO is highly reactive and its reaction with hemoglobin is considered to be a major route for its detoxification (Wennmalm *et al.* 1992) in humans. NO reacts rapidly with oxyhemoglobin forming nitrate and methemoglobin [$Hb(Fe^{+3})$]. This route for metabolism of NO, with nitrate being recycled, would be advantageous to the hypoxic plant cell, exposed to conditions of prolonged soil waterlogging, where

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Abbreviations: cNR - cytosolic nitrate reductase; DTT - dithiothreitol; EDTA - ethylenediaminetetra-acetic acid; GC - guanine-cytosine; HEPES - N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid; Ni-NOR - nitrite-nitric oxide reductase; NSHb - non-symbiotic hemoglobin; PB - Pusa Baisakhi; RT-PCR - reverse transcriptase polymerase chain reaction; Tm - melting temperature.
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nitrates would be severely depleted. Hb(Fe^{+3}) can be reduced to Hb(Fe^{2+}) *via* NADH-dependent reductases (Poole 1994) and this reaction would provide an additional NAD^+ for glycolysis.

Corpas *et al.* (2004) have reported at least nine routes for the synthesis of NO in plants. One of the mechanism is through the involvement of an enzyme variously mentioned as NO synthase (Barroso *et al*. 1999), nitric oxide like synthase enzyme (Kim *et al.* 2006) or NO associated protein (Gas *et al*. 2009). Stöhr *et al.* (2001) suggested the involvement of a plasma membrane bound nitrite-nitric oxide reductase (Ni-NOR) as well as cytosolic-nitrate reductase (cNR) (Yamasaki *et al*. 1999). Nitrate reductase is activated upon exposure of plant roots to hypoxia and nitrate (Botrel and Kaiser 1997), and it consumes 2 mol of NADH, without oxygen consumption, per mol of NO produced. NO produced in these reactions does not accumulate due to hypoxia induced non-symbiotic hemoglobins, which are reported to remain in the oxyhemoglobin form, even at oxygen tensions two orders of magnitude lower than necessary to saturate cytochrome *c* oxidase. They act as NO dioxygenases converting NO back to nitrate, consuming NAD(P)H in the process (Igamberdiev *et al*. 2005).

Comprehensive studies on physiological and biochemical aspects of waterlogging have been done in cereal crops like maize, rice and some aquatic grasses and weeds. Mung bean, being a rainy season pulse crop, experiences large scale waterlogging during the early vegetative phase of its growth. We have earlier reported that under waterlogging stress comparatively tolerant genotypes *Vigna luteola* and T 44 were able to retain higher relative water content, membrane stability, chlorophyll and sugar contents and sucrose synthase and alcohol dehydrogenase activities than the susceptible genotype Pusa Baisakhi (Sairam *et al.* 2009). The present investigation, therefore, has been planned to validate the role of non-symbiotic hemoglobin and NO interaction, and the underlying molecular mechanism in imparting hypoxia tolerance by using above mentioned tolerant and susceptible mung bean genotypes, and one wild *Vigna* species at early vegetative stage.

Materials and methods

The experiment was conducted with two cultivated mung bean [*Vigna radiata* (L.) Wilczek] genotypes (tolerant T 44 and susceptible Pusa Baisakhi, PB), and *Vigna luteola* (Jacq.) Benth., a wild *Vigna* sp., highly tolerant to waterlogging. Seeds were procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi, Indian Institute of Pulse Research, Kanpur and National Bureau of Plant Genetic Resources, New Delhi. Sowing was done in earthen pots filled with clay-loam soil and farm yard manure in 3:1 ratio during the summer-rainy season. Pots were supplied with recommended dose of N, P and K. Before sowing, seeds were treated with the *Rhizobium* culture. Waterlogging treatment was given by placing pots with 25-d-old plants in plastic troughs filled with water to a height just 1 to 2 cm below the soil level in the pots. Treatments consisted of control, 2, 4, 6 and 8 d of waterlogging, and recovery after 4 d. Because plants of susceptible genotypes PB waterlogged for 8 d showed more than 75 % mortality during recovery. Recovery was uniformly studied in all the genotypes after 6 d of waterlogging. NO production and NR activity were assayed in 8 replicates. The design of the experiment was completely randomized (CRD) and data was analyzed by factorial CRD.

Nitrate reductase activity was measured according to Hageman and Flesher (1960) with little modification. Enzyme extraction was done by pulverizing weighed root material in liquid nitrogen in a pre-chilled mortar, followed by grinding with 100 mM phosphate buffer (pH 7.5), 4 mM cysteine, 5 mM EDTA and 3 % bovine serum albumin. Homogenate was centrifuged (refrigerated centrifuge model *3K 30*, *Sigma*, Osterode, Germany) at 4 °C for 20 min at 12 000 *g*. Supernatant was used as the source of enzyme. Assay mixture consisted of 0.5 cm^3 enzyme extract, 0.2 mM phosphate buffer (pH 7.5), 50 mM potassium nitrate, 0.68 μM NADH. Incubation was done at 30 °C. Reaction was stopped by adding 0.1 cm^3 zinc acetate (1.0 M) solution and 1.9 cm^3 90 % ethanol. In blank 0.1 cm^3 zinc acetate (1.0 M) solution and 1.9 cm^3 90 % ethanol were added prior to the addition of enzyme. The contents were centrifuged at 5 000 *g* for 10 min to remove the precipitate. Supernatant was used for colour development with 1 cm^3 each of sulfanilamide (1 % in 1 M HCl) and naphthylethylene diamino dihydrochloride (0.02 % aqueous solution). After 15 min absorbance was recorded spectrophotometrically (*Specord Bio-200*, *AnalytikJena*, Jena, Germany) at 540 nm against blank.

NO production was done by a semi-quantitative method according to Moller and Palmer (1982) and Stöhr *et al.* (2001). The assay is based on the reduction of NO_2^- to NO by plasma membrane fraction, possibly caused by the enzyme nitrite-nitric oxide reductase. For preparation of crude membrane fraction, fresh leaf tissue was cut into pieces and grounded in pre-chilled mortar and pestle with ice cold extraction buffer consisting of 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 3 mm Na_2EDTA, 10 mM ascorbic acid and 5 mM diethyl dithiocarbamate. Homogenate was filtered through 4 layers of nylon/ cheese cloth and centrifuged at 10 000 *g* for 10 min at 4 °C. For the isolation of crude membrane fraction the supernatant was twice centrifuged at 25 000 g for 20 min at 4 °C. The pellet was suspended in suspension buffer made up of 5 mM K-phosphate buffer, pH 7.8, 250 mM sucrose and 3 mM KCl. The crude membranes extract was stored at -70 °C. For NO assay 100 μg protein was incubated at 30 °C in 0.45 cm^3 of 0.1 M Hepes-KOH

(pH 6.0). The reaction was started by addition of $NaNO_2$ (1 mM) and cytochrome *c* (0.3 mg cm^{-3}) reduced with ascorbate (40 mM) to give a final volume of 0.5 cm^3. The assay mixture was flushed with nitrogen before adding protein, and incubated by shaking at 30 °C for 3 h in glass vials with screw tops. For NO detection, pre-cut filters (3 mm, *Whatman No. 10*) were soaked in a solution of 5 % sulfanilamide and 0.2 % N-(1-naphthyl)-ethylene-diamine dihydrochloride in methanol (Nims *et al.* 1996), air-dried and used immediately for the assay. The filters were placed into the screw tops of the vials so that only gaseous NO had access and interference by NO_2^- was avoided. After incubation, each filter was extracted with 2 cm^3 methanol for 30 min at room temperature and the absorbance of the solution was determined at 463 nm against methanol. The genotypic variation was also on expected lines. Protein estimation was done according to Bradford (1976).

For gene expression studies 25-d-old plants were subjected to waterlogging for 24 h, and root samples were harvested from control and treated plants. Total RNA from root tissue was extracted using *Trizol* reagent (GibcoBRL), as per the recommendations of manufacturer. DNA contamination was removed from the RNA samples using DNase I (*Qiagen*, Maryland, USA). Total RNA (1 μg) was reverse transcribed using gene specific degenerate primers and *Qiagen* one step RT-PCR kit. PCR conditions were standardized using gene-specific primers for tubulin. Linear amplification for semi-quantitative RT-PCR was obtained with 35 cycles. Reactions were conducted using *My Genie 32* thermal block PCR (*Bioneer*, Daejeon, South Korea) under the following conditions: initial activation for 15 min at 95 °C, reverse transcription for 30 min at 50 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, extension for 1 min at 72 °C, final extension for 10 min at 72 °C. The amplification products were electrophoresed on 1.2 % agarose gel at 120 V in TBE buffer (0.4 M Tris-borate + 1 mM EDTA, pH 8.0) using known concentration DNA ladders. Gels were stained with ethidium bromide and visualized on *Uvi Pro* gel documentation system (*Uvitec*, Cambridge, UK).

Nucleotide sequences for *class 1 non-symbiotic hemoglobin* and *cNR* genes were obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The *Basic Local Alignment Search Tool* (Altschul *et al.* 1997; http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify the homologs of candidate genes. For RT-PCR expression analysis and cloning of cDNAs, the following oligonucleotide primers were designed manually, and oligo quality (to avoid primer dimmer, self dimer, *etc*.), guanine-cytosine (GC) % and melting temperature (Tm) were analyzed by using *Oligoanalyzer 3.0* tool (http://www.idtdna.com/analyzer/applications/oligoanalyzer/, *Integrated DNA Technologies*, Coralville, USA). Primers for various genes were designed using the data base; *non-symbiotic-Hb* - *Arabidopsis thaliana* (Trevaskis *et al*. 1997), *Medicago sativa* (Seregelyes *et al.* 2000) and *Glycine max* (Anderson *et al.* 1996), cNR - *Glycine max* (Wu *et al.* 1995) and tubulin - *Vigna radiata* (Raha *et al.* 1987). Sequences for various primers are listed in Table 1.

RT-PCR amplified cDNAs were fractionated on agarose gel and purified. The purified cDNAs for each gene were cloned into *pTz57R/T* vector and transformed into *E. coli* (strain DH5α) cells. DH5α cells transformed with recombinant plasmids were selected based on antibiotic resistance as well as α-complementation method (Fig. 1). Ampicillin resistant putative recombinants were selected for plasmid isolation from transformed *E. coli* cells (Fig. 2). Restriction analysis was carried out by using *Kpn I* and *Hind III* enzymes flanking the cloning site of the vector pTz57R/T (Fig. 3*A*,*B*) to confirm the presence of cloned insert cDNA. Cloned insert cDNA in the pTz57R/T vector was sequenced by dideoxy chain termination method (Sanger *et al.* 1977) using T7 and SP6 primers.

Table 1. Sequence and characteristics of used primers. F - forward, R - reverse.

Primer name	Sequence	GC content [%]	Tm [°C]	Amplicon size [bp]
cNR - F	TGAACATCACCACGAGAGGT	45.8	60.4	486
cNR - R	CAAGTGTGCCATCCATGTTC	52.8	60.4	
NSHb - F	GTTTCWCKGAAGARCAAGAAGCTC	41.6	60.3	428
NSHb - R	GGCAYYGACCARCTGATCATAAGC	45.8	57.8	
Tubulin - F	CTTGACTGCATCTGCTATGTTCAG	45.8	55.5	422
Tubulin - R	CCAGCTAATGCTCGGCATACTG	54.5	58.4	

Results

Shifting of plants to waterlogging condition increased the NO production activity up to 6 d in the roots of *Vigna luteola* (highly tolerant) and T 44 (tolerant), and up to 4 d in PB (susceptible) (Table 2). From 6th day onwards, decline in NO production was observed in PB, while in *V. luteola* and T 44 the decline was observed on 8th day of waterlogging. However, even on 8th day of waterlogging the NO production was 4 and 2 times higher in

tolerant genotypes than in the respective untreated plants. Recovery after 4 d of release of stress showed uniform decline in NO production in all three genotypes.

Table 2. Nitric oxide production [nmol mg^{-1}(protein) min^{-1}] in the roots of *Vigna luteola and Vigna radiata* T44 and PB during waterlogging lasting 0, 2, 4, 6 and 8 d and 4* d after recovery of plants treated for 6 d. Means ± SE, n = 8.

Time [d]	*V. luteola*	T 44	PB
0	6.38±0.11	5.60±0.09	5.19±0.02
2	14.55±0.17	10.80±0.01	7.07±0.09
4	21.90±0.00	13.81±0.09	8.97±0.03
6	40.51±0.07	29.31±0.13	8.66±0.03
8	28.42±0.03	13.48±0.05	6.45±0.04
4*	11.87±0.02	6.19±0.02	5.71±0.11

Table 3. Nitrate reductase activity [μmol mg^{-1}(protein) min^{-1}] in the roots of *Vigna luteola and Vigna radiata* T44 and PB during waterlogging lasting 0, 2, 4, 6 and 8 d and 4* d after recovery of plants treated for 6 d. Means ± SE, n = 8.

Time [d]	*V. luteola*	T 44	PB
0	6.50±0.36	5.50±0.52	5.00±0.38
2	8.17±0.52	7.17±0.26	5.83±0.57
4	9.17±0.65	9.00±0.40	4.50±0.69
6	7.17±0.58	7.50±0.42	3.50±0.55
8	6.50±0.54	4.67±0.31	2.83±0.69
4*	7.50±0.37	7.00±0.50	4.67±0.41

Nitrate reductase activity in the roots increased under waterlogging stress in all the genotypes (Table 3). In T 44 and *V. luteola* NR activity increased up to 4 d of waterlogging, while in PB highest NR activity was observed on 2nd day of waterlogging stress. Under all the waterlogging treatments highest NR activity were observed in *V. luteola*, followed by T 44 and lowest in PB. NR activity declined after 6 and 8 d of waterlogging in *V. luteola* and T 44. Recovery of 6-d waterlogged plants showed uniform increase in activity in all the genotypes; and in all the genotypes except PB, NR activity was greater than in the control plants.

Total RNA isolated from control and waterlogged mung bean genotypes was run on agarose gel electrophoresis (Fig. 1) to check the quality of RNA before running RT-PCR reactions. The results of RT-PCR analysis of *cNR* gene yielded an amplicon of about 480 bp in all the three genotypes (Fig. 2*A*). *cNR* mRNA expression was greater in waterlogged roots of *V. luteola* and T 44, while low expression was observed in control plants of *V. luteola* and T 44, and control and waterlogged plants of PB.

In the case of *NSHb* gene expression an amplicons of size of 430 bp was observed only in the waterlogging treated *V. luteola* and T 44 genotypes (Fig. 2*B*). No expression was observed in control plants of all the three genotypes and waterlogged roots of PB. DNA-PCR, however, confirmed the presence of gene in all the three genotypes (data not reported). This confirms that the *NSHb* expression is waterlogging inducible and also based on the extent of resistance nature of genotype(s). The tubulin expression was almost constant in all the genotypes, and did not change under control and waterlogging conditions (Fig. 2*C*).

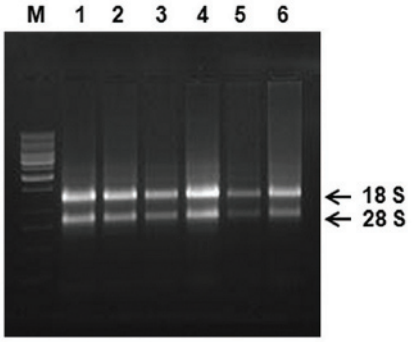

Fig. 1. Agarose gel electrophoresis of total RNA isolated from control and waterlogged mung bean genotypes (M - 1 kb ladder, 1 - control *V. luteola,* 2 - treated *V. luteola,* 3 - control T 44, 4 - treated T 44, 5 - control Pusa Baisakhi, 6 - treated Pusa Baisakhi).

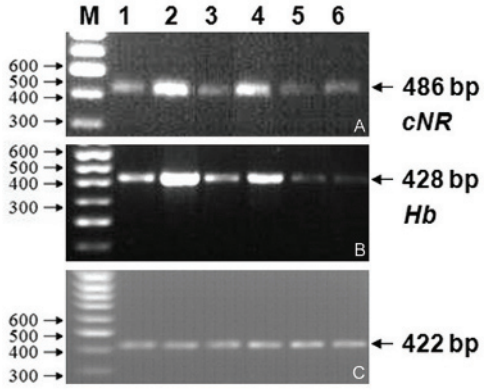

Fig. 2. RT-PCR expression analysis of cytosolic *nitrate reductase* (*cNR)* (*A*), *non-symbiotic hemoglobin* (*Hb*) (*B*) and *tubulin* (*C*) genes under waterlogging and control conditions (M - 1 kb ladder, 1 - control *V. luteola,* 2 - treated *V. luteola,* 3 - control T 44, 4 - treated T 44, 5 - control Pusa Baisakhi, 6 - treated Pusa Baisakhi).

E. coli (strain DH5α) cells were transformed with recombinant plasmids. Competent cells transformed with ligation products were plated on LA+Amp100+X-gal+IPTG plates (LB agar plate supplemented with ampicilin 100 mg dm^{-3} + 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside + isopropyl β-D-1-thiogalacto-pyranoside). The white coloured colonies shows recombinants, while blue/dark colonies showed cells transformed with self ligated pTZ57R/T. Figs. 3 and 4 show isolation of recombinant plasmids from *E. coli* cells, and restriction analysis of recombinant plasmids

with *Kpn* I/*Hind* III enzymes and fractionated on 1 % agarose gel. *cNR* gene specific primers yielded partial gene coding sequences of 479, 477 and 478 bp in *V. luteola,* T 44 and PB, respectively. Partial nucleotide sequences for *cNR* of the three genotypes are given below:

Vigna luteola
GAGCTTCTGTCCCCGGATCACGGCTTCCCCGTTAGGATGATAATACCCGGTTTCATTGGCGGCCGCATGGT
GAAATGGTTGAAGCGCATCGTTGTCAGTAACCAGCAATCTCAAAGTCATTACCATTACAAGGATAACAAG
CTATTTCCATCTCACGTTGACGCCGAACTTGCCAATGAAGAAGATTGGTGGTACAAGCCAGAGTATATTA
TCAACGAGGTCAACATAAACTCCGTCATAACAACTCCCTCCCACCAAGAGATCTTGCCCATCAACTCATG
GACAACTCAGATGCCATATTCCATGAGAGGCTACGCCTATTCCGGGGGGTGGGAGAAAGGTGACACGTGTG
GAGGTAACCCTGGACGGTGGTGAAACGTGGCAAGTGTGCAGTGTGGAGCGTCTGGAGAAACCGAACAAA
TATGGCAAGTACTGGTGTTGGTGTTTCTGGTCCTTGGAGGTGGAGGTGTTGGATATCAC

T 44
AAGTTTCTGTCCCCGGATCACGGCTTCCCCGTTAGGATGATAATACCCGGTTTCATTGGCGGCCGCATGGT
GAAATGGTTGAAGCGCATCGTTGTCAGTAACCAGCAATCTCAAAGTCATTACCATTACAAGGATAACAAG
CTATTTCCATCTCACGTTGACGCCGAACTTGCCAATGAAGAAGATTGGTGGTACAAGCCAGAGTATATTA
TCAACGAGGTCAACATAAACTCCGTCATAACAACTCCCTCCCACCAAGAGATCTTGCCCATCAACTCATG
GACAACTCAGATGCCATATTCCATGAGAGGCTACGCCTATTCCGGGGGGTGGGAGAAAGGTGACACGTGTG
GAGGTAACCCTGGACGGTGGTGAAACGTGGCAAGTGTGCAGTGTGGAGCGTCTGGAGAAACCGAACAAA
TATGGCAAGTACTGGTGTTGGTGTTTCTGGTCCTTGGAGGAGGAAATGTTAGGTATC

Pusa Baisakhi
ACGTTTCTGTCTCTGGATCACAGCTTCCCCGTTAGGATGATAATACCCGGTTTCATTGGCGGCCGCATGGT
GAAATGGTTGAAGCGCATCGTTGTCAGTAACCAGCAATCTCAAAGTCATTACCATTACAAGGATAACAAG
CTGTAACCATCTCACGTTGACGCCGAACTTGCCAATGAAGAAGATTGGTGGTACAAGCCAGAGTATATTA
TCAACGAGGTCAACATAAACTCCGTCATAACAACTCCCTCCCACCAAGAGATCAAGCCCATCCCCTCATG
GACAACTCAGATGCCATATTCCATGAGAGGCTACGCCTATTCCGGGGGGTGGGAGGGAGGTGACACGTGTG
GAGGTAACCCTGGACGGTGGTGAAACGTGGCAAGTGTGCAGTGTGGAGCGTCTGGAGAAACCGAACAAA
TATGGCAAGTACTGGTGTTGGTGTTAGTTGTCCTTGGAGGAGGAAATGTTAGGTATCA

NSHb gene specific primers yielded partial sequences of 418 and 423 bp only *in V. luteola* and T 44, respectively. Partial nucleotide sequences for *NSHb* of the two genotypes are given below:

Vigna luteola
AGGCTCTGGTGGTGAAGTCATGGAATGTCATGAAGAAGAATTCTGGAGAGTTGGGTCTCAAGTTTTTCTT
GAAAATATTTGAGATTGCTCCATCAGCTCAGAAATTGTTCTCATTCTTGAGAGATTCAACGGTTCCTTTGG
AGCAAAATCCCAAGCTCAAGCCCCATGCCGTGTCTGTTTTTGTAATGACCTGTGATTCAGCAGTTCAGCTG
CGGAAGGCCGGGAAAGTCACTGTCAGAGAATCAAACTTGAAAAAATTAGGTGCTACCCATTTTAGAACC
GGCGTAGCAAACGAGCATTTCGAGGTGACAAAGTTTGCACTGTTGGAGACCATAAAAGAAGCTGTACCA
GAAATGTGGTCACCGGCTATGAAGAATGCATGGGGAGAAGCTTATGATCAGCTGGTCGATGCCATTAA

T 44
AGTAGCTGGTGGTGAAGTCATGGAATGTCATGAAGAAGAATTCTGGAGAGTTGGGTCTCAAGTTTTTCTT
GAAAATATTTGAGATTGCTCCATCAGCTCAGAAATTGTTCTCATTCTTGAGAGATTCAACGGTTCCTTTGG
AGCAAAATCCCAAGCTCAAGCCCCATGCCGTGTCTGTTTTTGTAATGACCTGTGATTCAGCAGTTCAGCTG
CGGAAGGCCGGGAAAGTCACTGTCAGAGAATCAAACTTGAAAAAATTAGGTGCTACCCATTTTAGAACC
GGCGTAGCAAACGAGCATTTCGAGGTGACAAAGTTTGCACTGTTGGAGACCATAAAAGAAGCTGTACCA
GAAATGTGGTCACCGGCTATGAAGAATGCATGGGGAGAAGCTTATGATCAGCTGGTCGATGCCATTAAAT
AGT

Discussion

One of the problems faced by plants experiencing hypoxia/anoxia is increase of NAD(P)H/NAD(P) ratio, which adversely affects the glycolytic pathway, the only pathway which provides energy under anaerobiosis. To ameliorate this situation, plants use fermentative pathway employing lactic dehydrogenase and alcohol dehydrogenase for recycling of NAD(P)H to NAD(P). Earlier we have reported that roots of tolerant genotypes of pigeon pea (*Cajanus cajan*) and mung bean have higher contents of total-, reducing- and non-reducing sugars, activity of

sucrose synthase and alcohol dehydrogenase and level of respective genes expression (Kumutha *et al.* 2008, Sairam *et al.* 2009). However, glycolysis, followed by fermentation will most possibly lead to accumulation of toxic lactic acid, ethanol and CO_2 in the root tissues. Involvement of hypoxia-induced non-symbiotic hemoglobin and NO has been suggested as possible route for recycling of NADH to NAD in waterlogged plants (Ohwaki *et al.* 2005).

Waterlogging caused increase in both NO production as well as NR activity in *V. luteola* and T 44 with maximum at 6-d treatment, while not many changes were observed in PB. Even after 8 d of waterlogging tolerant genotypes showed approximately 3 times higher NO production than the unstressed plants. Dordas *et al.* (2003a,b) reported accumulation of NO and non-symbiotic hemoglobin in alfalfa root culture under hypoxia stress.

NR activity increased only up to 4th day of waterlogging and then declined. It was higher in *V. luteola* and T 44 than in PB. In roots, two distinct types of NR have been reported, one located in the cytosol (cNR) and the other attached to the plasma membrane and facing the apoplast (PM-NR) (Stöhr and Ullrich 1997, Stöhr and

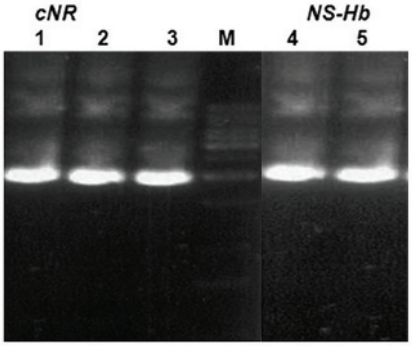

Fig. 3. Recombinant plasmid isolated from transformed *E. coli* cells (M - 1 kb ladder, 1 - *V. luteola cNR,* 2 - T 44 c*NR,* 3 - PBc*NR,* 4 - *V. luteola NSHb,* 5 - T 44 *NSHb).*

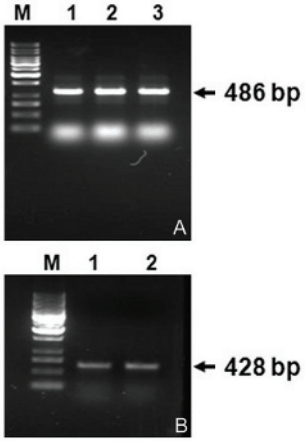

Fig. 4. Restriction analysis of recombinant plasmids with *Kpn* I /*Hind* III enzymes, and fractionated on 1 % agarose gel (*A* - *cNR* and *B* - *NSHb*).

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T44NR           -------------------------LSPDHGFPVRMIIPGFIGGRMVKWLKRIVVSNQQSQ 36
PB-NR           ---------------------------------MIIPGFIGGRMVKWLKRIVVSNQQSQ 26
VLNR            -------------------------LSPDHGFPVRMIIPGFIGGRMVKWLKRIVVSNQQSQ 36
X53603          SLMREVALDPSRDIILAYMQNGELLSPDHGFPVRMIIPGFIGGRMVKWLKRIVVSNQQSQ 298
GMU13987        SILREVALDPSRDIILAYMQNGEPLSPDHGFPVRMIIPGFIGGRMVKWLKRIIVTTDQSQ 300
                                                  ******************:*:.:***

T44NR           SHYHYKDNKLFPSHVDAELANEEDWWYKPEYIINEVNINSVITTPSHQEILPINSWTTQM 96
PB-NR           SHYHYKDNKLYPSHVDAELANEEDWWYKPEYIINEVNINSVITTPSHQEIKPIPSWTTQM 86
VLNR            SHYHYKDNKLFPSHVDAELANEEDWWYKPEYIINEVNINSVITTPSHQEILPINSWTTQM 96
X53603          SHYHYKDNKLFPSHVDAELANEEDWWYKPEYIINEVNINSVITTPSHQEILPINSWTTQM 358
GMU13987        NYYHYKDNRVLPSHVDAELANAQAWWYKPDYIINELNINSVITTPCHEEILPINSWTTQM 360
                .:******:: ********** : *****:*****:*********.*:** ** ******

T44NR           PYSMRGYAYSGGGRKVTRVEVTLDGGETWQVCSVERLEKPNKYGKYWCWCFWSLEEEMLG 156
PB-NR           PYSMRGYAYSGGGREVTRVEVTLDGGETWQVCSVERLEKPNKYGKYWCWC---------- 136
VLNR            PYSMRGYAYSGGGRKVTRVEVTLDGGETWQVCSVERLEKPNKYGKYWCWCFWSLEVEVLD 156
X53603          PYSMRGYAYSGGGRKVTRVEVTLDGGETWQVCSVERLEKPNKYGKYWCWCFWSLEVEVLD 418
GMU13987        PYFIRGYAYSGGGRKVTRVEVTLDGGETWQVCTLDCPEKPNKYGKYWCWCLWSVEVEVLD 420
                ** :**********:******************:::  *************

T44NR           I------------------------------------------------------------ 157
PB-NR           ------------------------------------------------------------
VLNR            I------------------------------------------------------------ 157
X53603          ILGAKEIAVRAWDEAQNTQPEKLIWNTMGMINNCWFRVKTNVCKPKKGEIGIVFEHPTQP 478
GMU13987        LLGAREIAVRAWDEALNTQPEKLIWNVMGMMNNCWFRVKTNVCRPHKGEIGIVFEHPTQP 480
```

Fig. 5. Comparison of c*NR* deduced amino acid sequence by *CLUSTAL W* (*v. 1.83*) multiple sequence alignment (* show conserved amino acid; *colored/dark* letters show amino acid polymorphisms).

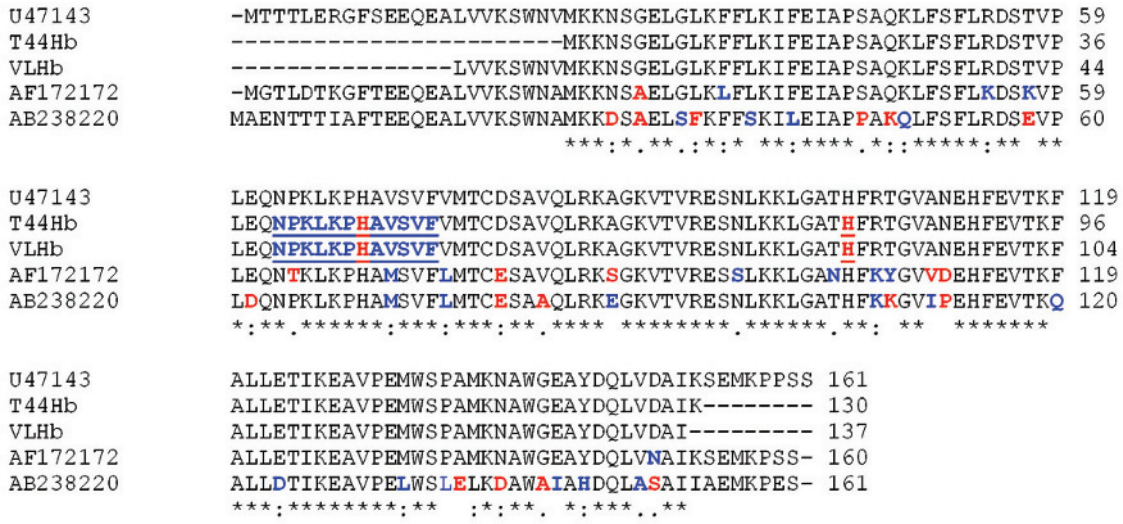

Fig. 6. Comparison of deduced amino acid sequence of NS-Hb by *CLUSTAL W* (*v. 1.83*) multiple sequence alignment (* show conserved amino acids; *colored/bold* letters show amino acid polymorphisms; *underlined* amino acid residues indicate the conserved oxygen binding active site).

Mäck 2001). A 2.5-fold activation of cNR during exposure of plant roots to hypoxia have been reported by Botrel and Kaiser (1997), with nitrite reduction being suppressed (Botrel *et al*. 1996). It has been suggested that a side-reaction of cNR is the reduction of nitrite to NO with NADH as an electron donor, probably catalyzed by the same molybdenum cofactor-containing domain as in nitrate reduction (Yamasaki *et al*. 1999). Results of Stöhr *et al.* (2001) and our results revealed a parallel increase in NR activity and NO production. Thus it seems plausible that both cNR and nitrite-nitric oxide reductase (Ni-NOR) may be involved in NO synthesis under hypoxic conditions, particularly in T 44 and *V. luteola*.

Waterlogging induced *cNR*-mRNA expression in the roots of *V. luteola* and T 44, while low expression was observed in PB. This correlates with NR activity in root tissue. RT-PCR amplified *cNR* cDNAs of *V. luteola*, T 44 and PB were sequenced and about 479, 477, 478 bp of partial coding sequence, and 157, 157, 136 amino acid sequences were obtained, which were compared with *Phaseolus vulgaris Genbank* acc. No. X53603 (Hoff *et al.* 1991) and *Glycine max Genbank* acc. No. GMU13987 (Wu *et al.* 1995) by *Clustal W* (*v. 1.83*) multiple sequence alignment data base (Fig. 5). These comparisons confirmed that the obtained PCR bands were of the *cNR* gene. *V. luteola* showed 99 % similarity with *P. vulgaris*, whereas T 44 and PB showed 98 and 94 % similarity, respectively. All three genotypes showed 80 - 85 % similarity with *G. max*. *P. vulgaris* showed 85 % similarity with *G. max*, which suggests that *V. luteola* and T 44 cNR are more closely related with *P. vulgaris* in sequence similarity than with *G. max*. The deduced protein sequence of all three genotypes pertains to eukary_NR_Moco, molybdopterin binding domain of eukaryotic NR, which belongs to SO family Moco super family. The Moco domain lies at the N-terminus of the assimilatory NR, which catalyzes the reduction of nitrate to nitrite. All known members of this family contain one pterin cofactor, and catalyze the transfer of oxygen to or from a lone pair of electrons in the substrate. Part of the coordination of Mo is a cysteine ligand.

In the present study, the gene expression of *NSHb* was observed only in the waterlogged roots of *V. luteola* and T 44, whereas no expression was observed in the control plants of all three genotypes as well as in waterlogged roots of PB. Expression of non-symbiotic hemoglobin only in the roots of waterlogged T 44 (tolerant genotype) and *V. luteola* (highly tolerant wild genotype) further confirms that it plays an important role in imparting waterlogging tolerance to these genotypes. Watts *et al.* (2001) cloned *Arabidopsis NSHb* cDNA under stress induction. Stress-induced hemoglobins have also been implicated in regeneration of NAD^+ during hypoxia (Hill 1998) based on the observations that alcohol dehydrogenase activity and CO_2 production is reduced under hypoxia in maize cells constitutively expressing barley hemoglobin (Sowa *et al.* 1998). Similarly transformed alfalfa root culture lines constitutively expressing barley hemoglobin maintained root growth during hypoxic treatment, whereas wild-type and lines with suppressed stress-induced hemoglobin expression had slower root growth (Dordas *et al.* 2003a, 2004).

NSHb cDNAs were cloned and 486 and 423 bp of partial nucleotide sequences, and 130 and 137 deduced amino acids sequences were obtained in *V. luteola* and T 44, respectively, and analyzed by *ClustalW* data base using *Medicago sativa* (*Genbank* acc. No. AF172172; Seregelyes *et al.* 2000), *G. max* (acc. No. U47143; Anderson *et al.* 1996) and *Lotus corniculatus* (acc. No. AB238220; Uchiumi *et al.* 2002) (Fig. 6). The comparison results confirmed that the obtained PCR bands were of the *NSHb* gene. The two genotypes

showed nucleotide sequences similarity of 98 % with each other, whereas with *G. max* up to 99 % similarity. In the case of deduced amino acid sequences, 100 % similarity was observed with *G. max*, while only 87 % similarity was observed with *Medicago sativa*. Using *Blast* tool, conserved domains were identified, which confirmed that the bands obtained in T 44 and *V. luteola* belongs to globin (cd01040). Globins are heme proteins, which bind and transport oxygen in microorganisms and animals and also plant leghemoglobins

Regeneration of nitrate is essential under nitrate limiting conditions of anaerobic roots for the continuation of NSHb-NO cycle. It has been suggested that oxyhemoglobin [$Hb(Fe^{2+})$-O_2] can donate negatively charged dioxygen to NO forming NO_3^- and methemoglobin (Di Iorio 1981). The reduction of methemoglobin to hemoglobin by a methemoglobin reductase which can occur in the nodules of leguminous plants (Topunov *et al*. 1980), or by endoplasmic reticulum cytochrome b_5 reductase (Hagler *et al.* 1979) or dihydrolipoamide dehydrogenase (Igamberdiev and Hill 2004).

Oxygen deficiency induced class 1 non-symbiotic hemoglobin and NO (*via* cNR and/or Ni-NOR). This route may serve as an alternative to fermentation pathway by facilitating consumption of reducing potential and regeneration of NAD, essential for the glycolytic pathway, which is the only energy generating pathway during the oxygen deficiency. Both cNR and Ni-NOR require reducing potentials in the form of NADH or other reduced intermediates, which further require NADH for their reduction, for the synthesis of NO_2^- and NO. Further reduction of methemoglobin ($HbFe^{3+}$) to hemoglobin ($HbFe^{2+}$), which is essential for the formation of oxy-hemoglobin complex and for the regeneration of NO_3^- from NO, requires reducing potential in the form of NADH. It is, thus evident that NSHb-NO interaction may acts as an alternative pathway for oxidation of NADH to NAD, and consequently of fermentation pathway and thereby lowering the possibility of accumulation of toxic CO_2, ethanol and lactic acid and thus could be one of the physiological factors providing tolerance to *Vigna luteola* and T 44 (*V. radiata*) under waterlogging stress.

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