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ISOLATION, MOLECULAR CHARACTERIZATION AND EXPRESSION OF NITRITE REDUCTASE GENE FROM EGYPTIAN SPINACH

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ABSTRACT

The nitrite reductase (NiR) gene was isolated, cloned and sequenced from nitrate-induced leaves of the Egyptian Spinacia oleracea seedlings. The sequence of the isolated NiR gene has a 1788 bp open reading frame. The nucleotide sequence of the isolated NiR gene was submitted to the NCBI GenBank database under the accession number MH729808. The deduced protein sequence consisted of 595 amino acid residues with a molecular weight of 66.5 kDa and an isoelectric point of 6.37. The sequence had a 96% homology with the predicted ferredoxin-nitrite reductase sequence, chloroplastic (Spinacia oleracea) and was highly conserved. The highest amino acid percentage belonged to leucine in the predicted NiR protein, which has a higher preference for the α-helix structure. RT-PCR analysis showed that the expression levels of NiR had increased after 2 hrs of potassium nitrate treatment. The NiR protein had a high activity towards $NaNO_2$ substrate after a 3 hr-induction period with potassium nitrate.

KEYWORDS: Accession Number MH729808, Nitrate Treatment, Nitrite Reductase (NiR) Activity, Real Time-PCR & Spinacia Oleracea

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Original Article

1. INTRODUCTION

Majority of the absorbed NO_2 by plants is converted to nitrate and nitrite which is then assimilated into ammonia and converted into amino acids through the nitrate primary assimilatory pathway **(Morikawa et al., 2003; Takahashi et al., 2001)**. Nitrate is essential for plant growth and development as it is a major source of nitrogen. It produces amino acids needed for protein synthesis and other plants' vital processes **(Balotf et al., 2016)**. It is a signaling molecule that can incite the shoot growth, regulate the stomatal opening and relieve seed dormancy **(Wu et al., 2015)**.

The nitrogen assimilatory pathway contains three enzymes, nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS). NiR protein is found in the plastid, and it converts nitrite into ammonia **(Davenport et al., 2015)**. Nitrogen dioxide or nitrate can induce *NiR* expression **(Takahashi et al., 2001)**. *NiR* has other valuable functions in getting rid of excess nitrite. Nitrite produced in the nitrogen cycle is toxic to plant cells, affecting cell growth and regeneration. High NiR enzyme activity is an indicator of high

regenerative ability of the plant cells **(Han et al., 2010)**. During rice and cotton embryogenesis, *NiR* is vital in the nitrogen assimilation process (**Han et al., 2010)**. *NiR* has been isolated and cloned from spinach **(Back et al., 1988)**; Tobacco (**Kato et al., 2004; Kyaing et al., 2012)**; shoots of *Oryza stavia* L. seedling **(Terada et al., 1995)**; *Gossypium hirsutum* callus **(Han et al., 2010)**; Mulberry (*Morus* L.) **(Wang et al., 2015)** and Radish **(Wu et al., 2015)**. NiR protein is a monomer and has two redox centers: a siroheme-Fe center for nitrite reactivity and aniron-sulfur center which acts as an initial electron acceptor. The NiR protein is also composed of 3 domains, nitrite reductase ferredoxin-like domain, iron-siroheme binding site and 4Fe-4S clusters. Reduced ferredoxin acts as the electron donor, presents six electrons to nitrite in order to form ammonia **(Wu et al., 2015)**. *NiR* was induced using different concentrations of nitrate **(Privalle et al., 1989)**. Spinach (*Spinacea oleracea*) is an annual, edible green leafy vegetable **(Panda et al., 2017)**. It is a member of the subfamily Chenopodioideae (Chenopodiaceae) and is related to quinoa (*Chenopodium quinoa*), Swiss chard, and sugar beet (*Beta vulgaris* L.) **(Yamamoto et al., 2014)**. It is a cold tolerant, heat sensitive vegetable **(Zhao et al., 2018)**. It is rich in nutrients, antioxidants, flavonols, polyphenols, minerals and vitamins (A, B1, B2, B3, B6, C, E, K, and folic acid) and it is very healthy **(Panda et al., 2017)**. It has many biological activities like virus inhibition, anthelmintic activities, antioxidant activities, hepatoprotective activities as well as reducing the risk of breast cancer **(Metha & Belemkar, 2014)**. The Egyptian spinach plants (Baladi spinach) were used in the current study to isolate, clone and sequence the *NiR* gene. Moreover, the gene was subjected to further analysis to investigate the gene expression and protein activity using potassium nitrate over different time intervals.

2. MATERIALS AND METHODS

This study was carried out at the Molecular Manipulation and Gene Transfer lab (MMGT), Agricultural Genetic Engineering Research Institute (AGERI) and Agriculture Research Center (ARC), Giza, Egypt.

2.1 Plant Material

Baladi spinach seeds were purchased from the Agricultural Research Center (ARC), Giza, Egypt.

2.2 Germination and Treatment

The seeds were sterilized according to **Naderi et al. (2012)**. After sterilization, seeds were kept in water overnight, and they were placed in the refrigerator for 2 days after water removal. Finally, the seeds were placed in soil to initiate germination; 10 seeds per pot for 14-18 days. The Egyptian spinach seedling treatment was carried out according to **Privalle et al. (1989)** where, the seedlings were transferred into 20 mM KNO_3 solution over the time intervals 1, 2, 3, 4 and 24 hrs. For the control samples, some seedlings were placed in sterilized distilled H_2O. After treatment, the leaves were quickly frozen in liquid nitrogen, and then stored at -80 ^oC till RNA extraction.

2.3 RNA Extraction from Egyptian Spinach Seedlings

According to **Chomczynsk (1993),** total RNA was extracted from the control and treated Egyptian spinach seedlings using the TRIzol reagent (Ambion by Life Technology, REF. 15596026, USA). The RNA pellets were re-suspended in DEPC – treated water and stored in aliquots at -80 ^oC.

2.4 cDNA Synthesis using Isolated RNA

The cDNA was synthesized from both treated and control Egyptian spinach seedlings RNA using M-MLV Reverse Transcriptase enzyme (200 U/µl) (REF M1701, Promega, USA). They were synthesized according to the manufacturer's protocol of M-MLV enzyme. All cDNA was kept at -20 °C for subsequent PCR reactions.

2.5 Amplification of Actin and NiR Genes

Table 1: List of Used Primers

Primers	Primers Sequences
Actin Forward primer	5'- GAGACCTTTAATGTGCCCGC -3'
Actin Reverse primer	5'- ATCACGACCAGCAAGATCCA -3'
NiR Forward primer	5' ATGGCATCACTTCCAGTCAACAA-3'
NiR Reverse primer	5' CTACTCTGCCTCTTCCCTCTCCCTA-3'
Oligo dT primer	5' TTTTTTTTTTTTTTT 3'
Real-Time *NiR* Forward primer	5' TGTCATCGTCGAACAACAACA 3'
Real-Time *NiR* Reverse primer	5' CCTCCTTCAATACCCAAAACCC 3'

NCBI primer blast online software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and Primer3 web online software (http://primer3.ut.ee/) were used for designing the primers. All DNA primers used in PCR reactions were purchased from Operon Molecular for Life (Germany) (Table 1). The PCR reactions were carried out in BioRad PCR machine. Each reaction was prepared using Emerald Amp Max PCR Master Mix (Cat. #RR320A, Takara) according to kit manufacturer's protocol. In each PCR reaction, either actin or *NiR* primers were used. The PCR amplification conditions were as follows: initial denaturation at 95 °C for 5 min, then 35 cycles each at 94 °C for 1 min, 50 °C for actin or 60 °C for *NiR* for 45 sec, 72 °C for 1 min for actin or 2 min for *NiR*; followed by a final extension cycle at 72 °C for 5 min. The amplified products were tested on a 1.5% agarose gel.

2.6 Elution of Target Band from the Gel

The *NiR* gene expected band was eluted from the agarose gel using Gene JET gel extraction kit (Cat. K0691, Thermo Scientific, USA) according to the kit manufacturer's protocol. Then, the purified DNA was stored at -20 ^{o}C.

2.7 Cloning of Purified NiR DNA and Sequencing

The purified *NiR* DNA was cloned into pGEM-T easy vector. The ligation reactions were carried out according to the manufacturer's protocol of pGEM-T easy vector system (REF. A1360, Promega, USA). After the fragments were prepared, they were transformed into *E. coli* DH5α competent cells (Invitrogen, Cat. No. 18265-017, USA) according to **Sambrook et al. (1989)**. The pure Yield TM Plasmid Miniprep system kit (REF A1223, promega, USA) was used to isolate DNA from the transformed colonies. The isolated DNA was digested using restriction enzyme kit Fast Digest *EcoRI* (Cat. No. FD0274, Thermo Scientific, USA). After that, the DNA was sequenced. The sequencing reactions were performed on ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, USA) with T7 and SP6 primers in conjunction with ABI PRISM (310 Genetic Analyzer) using Sanger method (**Sanger et al., 1977**). The sequencing was carried out by Macrogen Company (South Korea).

2.8 Analysis of the Sequenced Fragment using NCBI Database

The National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) was used to analyze the sequence. Homology analysis at the DNA and protein level was performed for the obtained sequence using Basic local Alignment search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (**Altschul et al., 1997**).

2.9 Protein Sequence Analysis

The protein sequence and its open reading frame were detected using the NCBI Open Reading Frame Finder (OPR Finder) database (https://www.ncbi.nlm.nih.gov/orffinder/). This protein sequence was compared to other NiR proteins using

Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The amino acid composition of the protein was determined using Predict Protein Online tool (https://open.predictprotein.org). The molecular weight (M. wt) and the isoelectric point (pI) were deduced using Compute pI/Mw tool (https://web.expasy.org/compute_pi/). The secondary structure of the protein was predicted using The PSIPRED Protein Sequence Analysis online tool (http://bioinf.cs.ucl.ac.uk/psipred/). The 3D structure was built up by homology modeling using the Swiss-Model (https://swissmodel.expasy.org/).

2.10 Real Time PCR (RT-PCR)

Real time PCR reactions for *NiR* and actin (as reference gene) were carried out using the QuantiTect SYBR Green PCR Kit (Cat No.: 204141, QIAGEN, Germany) according to the manufacturer's protocol; the primers are shown in Table 1. The Mx3005P Real Time PCR system (Stratagen, USA) was used and the change in *NiR* expression for the treated spinach leaves was represented as the number of fold changes compared with the relative control plants. Relative gene expression ratios (RQ) between treated and control spinach leaves were calculated according to this formula $RQ = 2^{-\Delta\Delta CT}$ (**Livak & Schmittgen, 2001**).

2.11 Protein Extraction

The NiR enzyme was extracted according to **Takahashi et al. (2001)**. Bradford method was used to determine the total protein concentration in the protein **(Bradford, 1976)**.

2.12 Nitrite Reductase Bioassay

The nitrite reductase enzyme assay was carried out according to **Wrayand Fido (1990); Takahashi et al. (2001)**. The absorbance was then measured using infinite 200pro (Serial no. 1506002759, Tecan Austria GmbH) at 540 nm. Different known concentrations of sodium nitrite (0-0.3 mM) were used for constructing the standard curve to measure the remaining nitrite levels in the solution.

3. RESULTS AND DISCUSSIONS

3.1 Isolation of cDNA Encoding for NiR Gene from Baladi Spinach

In agreement with **Orea et al. (2001)**, Egyptian spinach seedling were treated with 20 mM KNO_3, over different time intervals (1, 2, 3, 4 and 24 hrs), then RNA was isolated from the control and treated leaves followed bycDNA synthesis from purified RNA. PCR reactions were performed using the synthesized cDNA and the actin and *NiR* primers. The actin PCR product (180 bp) was analyzed using 1.5% agarose gel electrophoresis (figure 1 A). The *NiR* PCR product (≈ 1788 bp) was eluted from the gel using gel Extraction kit (figure 1 B). It was found that the nitrate induced the expression of the *NiR*-gene. **Takahashi et al.** (**2001**) found that the plants with high NiR enzyme activity have high tolerance to NO_2. The *NiR* gene was involved in as well as controlled the nitrate assimilatory pathway in plants.

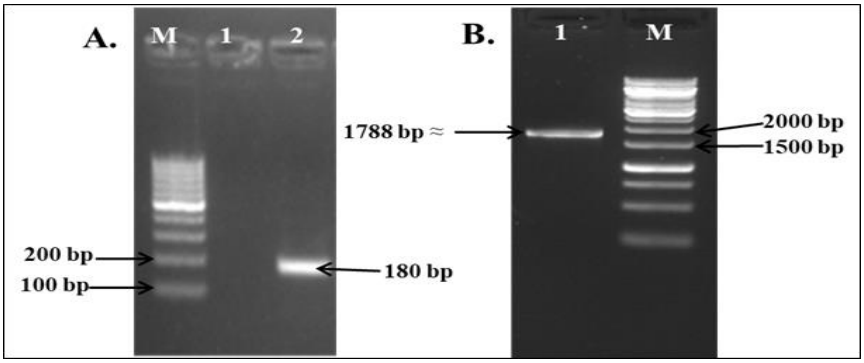


Figure 1: 1.5% Agarose Gel Electrophoresis Showing (a): PCR Using Actin Primers; Where Lane M Is 100 Bp DNA Markers (Genedirex); Lane 1 Is Negative Control; Lanes 2 Is Treated Spinach Seedlings. (b): The Target Band AFTER Purification. Lane 1 Is The Purified Band And Lane M Is 1 Kb Marker.

3.2 Cloning and Sequencing of Isolated NiR Gene

A) *NiR* complete cds 1788 bp

ATGGCATCACTTCCAGTCAACAAGATCATACCATCATCAACGACATTACTGTCATCGTCGAACAACAACAGAAG
AAGAAATAACTCATCAATTCGATGCCAGAAGGCGGTTTCACCCGCGGCAGAAACGGCTGCAGTGTCGCCGTCTG
TGGACGCGGCGAGGCTGGAGCCGAGAGTGGAGGAGAGAGATGGGTTTTGGGTATTGAAGGAGGAATTTAGGAG
TGGGATTAACCCAGCTGAGAAAGTTAAGATTGAGAAAGACCCAATGAAGTTGTTTATTGAGGATGGGATTAGTG
ATCTTGCTACTTTGTCAATGGAGGAAGTTGATAAATCTAAGCATAATAAGGATGATATTGATGTTAGACTCAAGT
GGCTTGGACTTTTCCATCGCCGTAAACATCACTATGGGAGATTCATGATGAGGTTGAAGCTGCCGAATGGGGTA
ACAACGAGTGAGCAGACACGGTACCTAGCAAGCGTGATCAAGAAGTACGGAAAAGATGGATGTGCGGATGTAA
CAACAAGGCAAAACTGGCAAATTAGAGGAGTTGTTCTGCCTGATGTGCCAGAGATCATCAAAGGGCTGGCATCC
GTGGGTCTTACCAGCTTACCCACTGGGGATGGCCAATTTAAGGAACCCCTTTCGGTAACCTTTCTCCAGGGAATG
ACTCTTCATGAAATTGTTGACACCCGACCTTTTACCAACCTAATTTCTCAATTTGTCACTGCCAATTCGCGTGGAA
ACCCTTCTATTACCAATCTGCCAAGGAAGTGGAATCCATGTGTTATTGGGTCCCATGATCTTTATGAGCATCCAC
ACATCAATGACCTTGCTTACATGCCTGCTACAAAGAATGGGAAATTCGGGTTTAATTTGTTGGTTGGAGGATTCT
TTAGCATCAAAAGATGTGAAGAGGCAATCCCACTAGACGCTTGGGTCTCAGCAGAAGATGTGGTTCCTGTATGC
AAAGCTATGCTTGAAGCTTTCAGGGACCTTGGCTTTAGAGGAAACAGGCAGAAGTGCAGAATGATGTGGCTTAT
TGATGAGCTTGGTATGGAAGCATTCAGGGGAGAGGTTGAGAAGAGAATGCCTGAGCAAGTTCTAGAAAGAGCA
TCCTCAGAAGAGCTGGTTCAGAAGGACTGGGAGAGAAGAGAATACTTAGGAGTTCACCCTCAGGAACAACAAG
GACTTAGCTTTGTGGGTCTCCACATTCCTGTGGGCCGTCTGCAAGCTGATGAGATGGAAGAGTTAGCCCGTATAG
CTGATGTGTATGGATCAGGGGAGCTCCGTCTGACAGTAGAGCAGAACATAATCATCCCAAATGTTGAAAACTCA
AAGATAGATTCACTACTAAACGAGCCTCTGTTAAAAGAGCGTTACTCCCCTGAACCACCCATCTTGATGAAGGG
GCTTGTGGCCTGTACGGGGAGCCAATTTTGTGGACAAGCCATTATCGAGACCAAGGCTAGGGCACTCAAGGTGA
CAGAAGAGGTACAACGACTAGTGTCTGTAACACGGCCTGTTAGGATGCATTGGACCGGGTGTCCTAATAGTTGT
GGTCAAGTACAAGTGGCTGATATTGGGTTCATGGGTTGCATGACTAGGGATGAGAACGGTAAGCCTTGTGAAGG
AGCTGATGTGTTTGTAGGAGGACGTATAGGAAGTGACTCGCATCTAGGAGACATTTACAAGAAGGCAGTCCCAT
GTAAAGATTTGGCGCCTGTTGTTGCTGAGATATTGATCAACCAATTCGGTGCTGTTCCTAGGGAGAGGGAAGAG
GCAGAGTAG

B) NiR protein 595 a.a (Frame +1)

MASLPVNKIIPSSTTLLSSSNNNRRRNNSSIRCQKAVSPAAETAAVSPSVDAARLEPRVEERDGFWVLKEEFRSGINPAE
KVKIEKDPMKLFIEDGISDLATLSMEEVDKSKHNKDDIDVRLKWLGLFHRRKHHYGRFMMRLKLPNGVTTSEQTRYL
ASVIKKYGKDGCADVTTRQNWQIRGVVLPDVPEIIKGLASVGLTSLPTGDGQFKEPLSVTFLQGMTLHEIVDTRPFTN
LISQFVTANSRGNPSITNLPRKWNPCVIGSHDLYEHPHINDLAYMPATKNGKFGFNLLVGGFFSIKRCEEAIPLDAWVS
AEDVVPVCKAMLEAFRDLGFRGNRQKCRMMWLIDELGMEAFRGEVEKRMPEQVLERASSEELVQKDWERREYLG
VHPQEQQGLSFVGLHIPVGRLQADEMEELARIADVYGSGELRLTVEQNIIIPNVENSKIDSLLNEPLLKERYSPEPPILM
KGLVACTGSQFCGQAIIETKARALKVTEEVQRLVSVTRPVRMHWTGCPNSCGQVQVADIGFMGCMTRDENGKPCEG
ADVFVGGRIGSDSHLGDIYKKAVPCKDLAPVVAEILINQFGAVPREREEAE*

Figure 2(a): Nucleotide Sequence of NiR Gene (1788 bp) with Gen Bank Accession Number MH729808. (b): The Deduced Coded 595 Amino Acid.

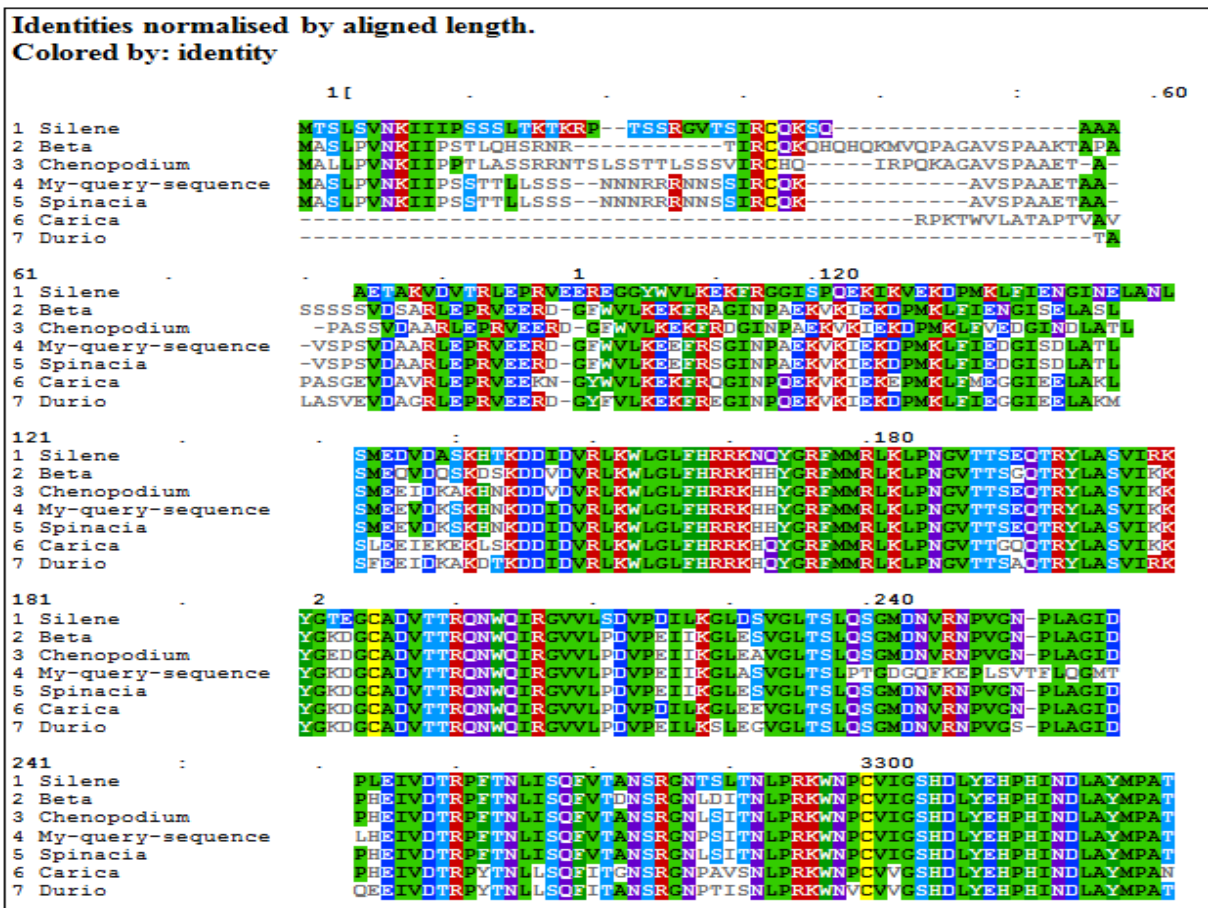
The eluted DNA was cloned. The isolated plasmids were digested using restriction digestion enzyme *Eco RI* to release the *NiR* gene fragment (≈1788 bp). The right colony was then sequenced using SP6 and T7 primers. From the sequencing results (Figure 2 A), the *NiR* gene length was 1788 bp. The *NiR* sequence was submitted to GenBank under

the accession number MH729808. The deduced amino acids sequence consisted of 595 amino acids (Figure 2 B). It was found that the Tobacco and Mulberry *NiR* lengths were 1764 bp and their amino acids were 587 and 586, respectively (**Kyaing et al., 2012; Wang et al., 2015**).

3.3 Computational Analysis

3.3.1 Analysis of Nucleotide Sequence of Spinach NiR Gene

Using Blast X database, the obtained *NiR* sequences howed high similarity with the published spinach nitrite reductase sequence at 96% identity. The NiR sequence also had different degrees of similarity with other NiR proteins such as *Chenopodium quinoa*(87%), *Beta vulgaris* (84%) and *Silene vulgaris*(80%). Alignment of the isolated *NiR* deduced amino acid sequence with the nitrite reductase amino acid sequence of other species identified the conserved regions (figure 3). Phylogenetic tree shows different degrees of similarity with other nitrite reductasesequences (figure 4). Phylogenetic analysis showed thatNiR was most closely related to the published NiR protein from Spinacia; Beta and *Chenopodium*. Spinach belongs to the *Amaranth*family and is related to beets and quinoa. The deduced amino acid sequence showed a high degree of similarity with other *NiR* sequences in higher plants. *NiR* sequence was well conserved in the plants as concluded from the phylogenetic distances (**Orea et al., 2001; Wang et al., 2015**). There were active residues and highly conserved binding sites identified after comparing the spinach *NiR* amino acid sequence with the other published *NiR* amino acid sequences such asthose pertaining to Mulberry (**Wang et al., 2015**).



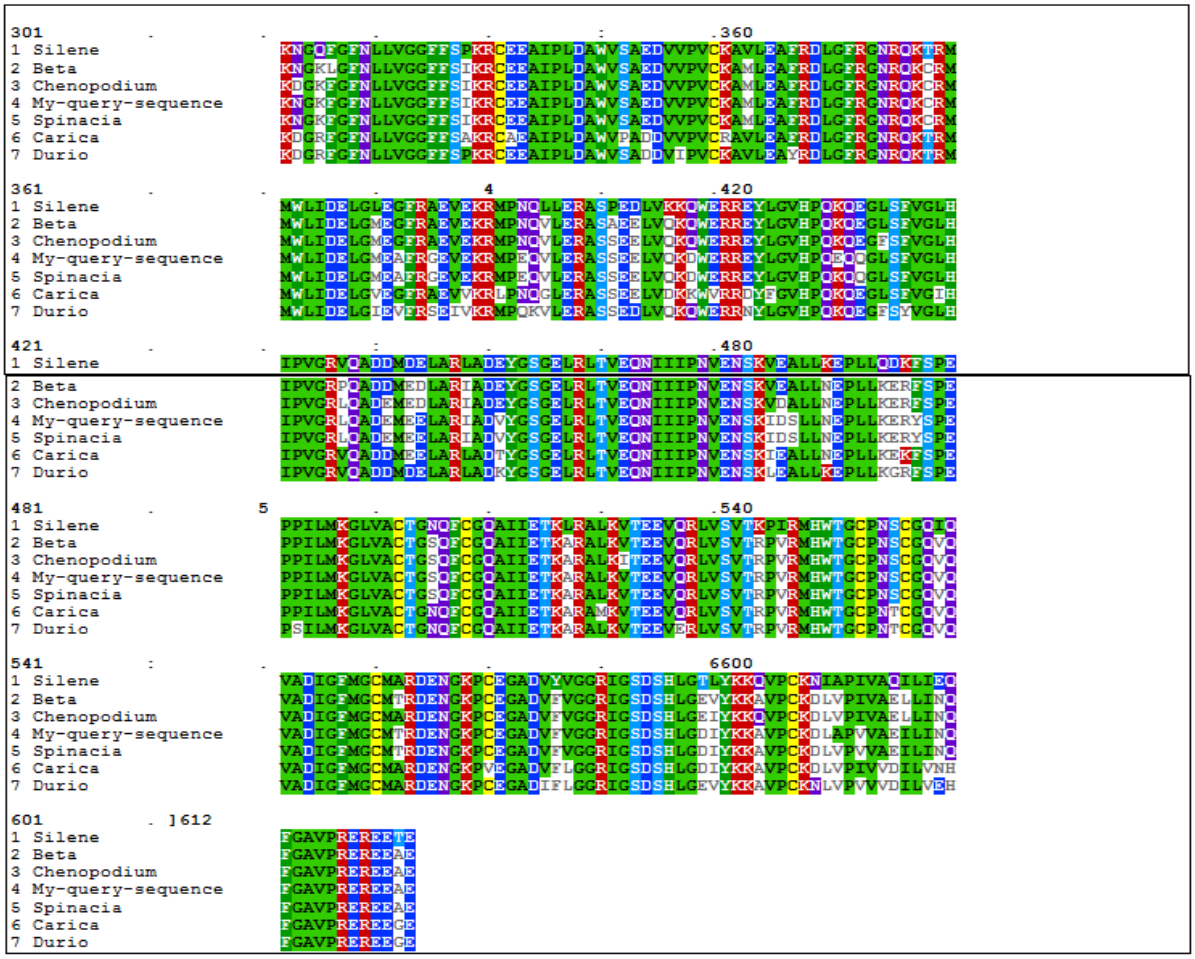


Figure 3: Multiple Sequence Alignment of the Isolated NiR Amino Acid Sequence with Nitrite Reductase of Several other Plants Including Silene (Silene Vulgaris, AGO67239.1), Beta (Beta Vulgaris, ADN97117.1), Chenopodium (Chenopodium Quinoa, XP_021754875.1), Spinacia (Spinacia Oleracea, XP_021866794.1), Carica (Carica Papaya, XP_021896718.1) and Durio (Durio Zibethinus, XP_022727235.1).

Figure 4: Phylogenetic Tree showing the Relatedness between a Number of Nitrite Reductase (NiR) Proteins including Silene (Silene Vulgaris, AGO67239.1), Beta (Beta Vulgaris, ADN97117.1), Chenopodium (Chenopodium Quinoa, XP_021754875.1), Spinacia (Spinacia Oleracea, XP_021866794.1), Carica (Carica Papaya, XP_021896718.1) and Durio (Durio Zibethinus, XP_022727235.1).

3.3.2 Analysis of the Deduced Protein Sequence

3.3.2.1 Primary and Secondary Protein Structure

The percentages of different amino acids in the deduced NiR protein were calculated using predict protein online software (https://open.predictprotein.org). The results showed that leucine (L) amino acid residue was the most dominant domain at

8.7% and tryptophan (W) was the least dominant one at 1.3% (Figure 5). This software showed that the deduced protein-was composed of 33.6% helix, 15.1% strand and 51.3% loop. Also, the results showed that the theoretical molecular weight (M.wt), and isoelectric point (PI) of the deduced amino acid sequence were 66.5 kDa and 6.37, respectively. For Mulberry, the NiR protein had a molecular weight of 65.4 kDa) Wang **et al., 2015**).

The secondary structure of the deduced protein was predicted as illustrated in Figure 6. It showed that the amino acids that form the α-helix were in the form of cylinders, the amino acids that had arrow shapes are those that form ß-sheets and coil structures was formed from amino acids in the form of lines in the protein. Leucine has a higher preference for the α-helix. Hydrophobic regions are important for protein stability and folding and also for creating non-aqueous setting for binding of the substrate and catalysis in many enzymes (**Brosnan and Brosnan, 2006**). Leucine has strong helix-forming power and it is also the most predominant amino acid in the inner helical regions of proteins. The aforementioned information show that lecucine may have a key role in the folding of protein molecules and it is one of the most frequent active site neighboring amino acid residue **(Chou and Fasman, 1973**).

%A: 6.2	%C: 2.2	%D: 5.0	%E: 8.4	%F: 3.5
%G: 7.4	%H: 2.0	%I: 5.7	%K: 5.9	%L: 8.7
%M: 2.9	%N: 4.4	%P: 5.7	%Q: 3.5	%R: 6.9
%S: 6.4	%T: 4.2	%V: 8.1	%W: 1.3	%Y: 1.5

Figure 5: The Percent of Different Amino Acid Residues.

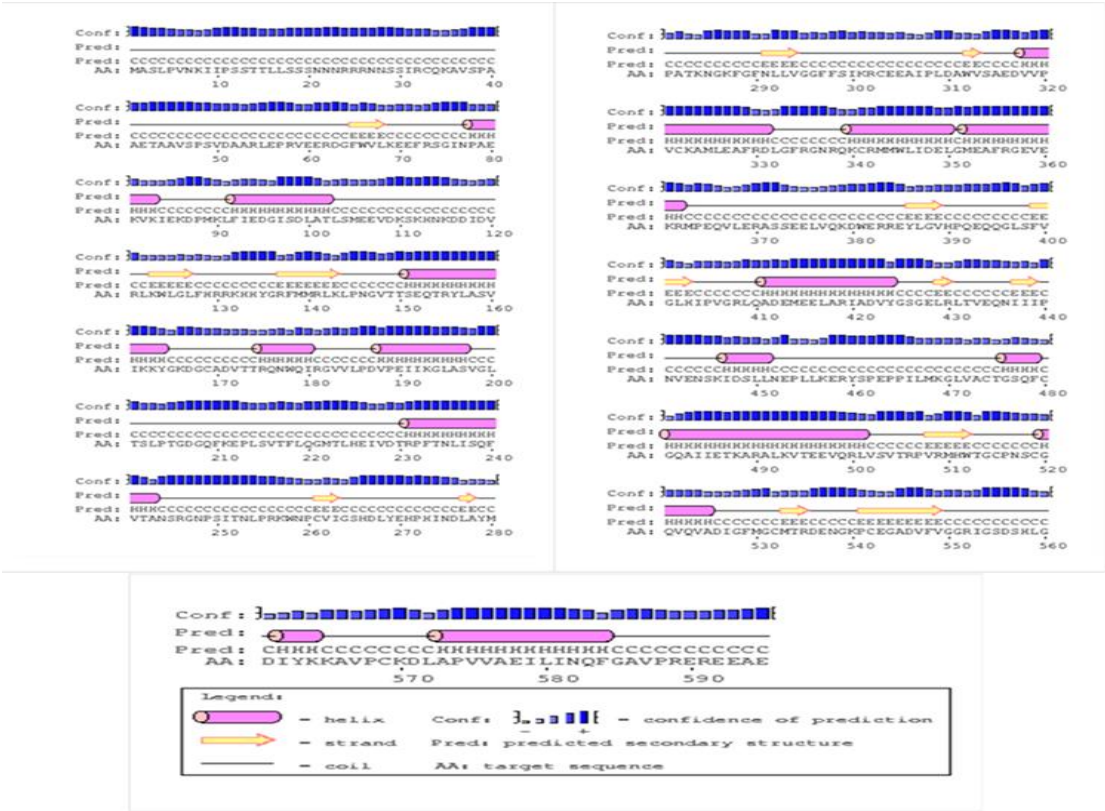


Figure 6: The Secondary Structure of Deduced NiR Protein.

3.3.2.1 3D structure of NiR Protein

Using the Swiss-Model (https://swissmodel.expasy.org/), the 2akj.1.A was used as a template to build the 3D structure of the isolated NiR protein as it has 96.44% sequence identity (Figure 7 A). It was found that the protein is a monomer and has 2 unique ligands 1 x SF4: 1 x SRM: SIROHEME (Figure7 B) and IRON/SULFUR CLUSTER (Figure 7 C). The hydrophobicity of the amino acids and the protein structure were illustrated in Figure 8. There was no trans-membrane domain detected. Protein-protein interaction was used to determine the hydrophobicity role. It was found that the location of the ligands corresponds with one of the strongest hydrophobic regions on the surface of the protein molecule (Young et al., 1994).

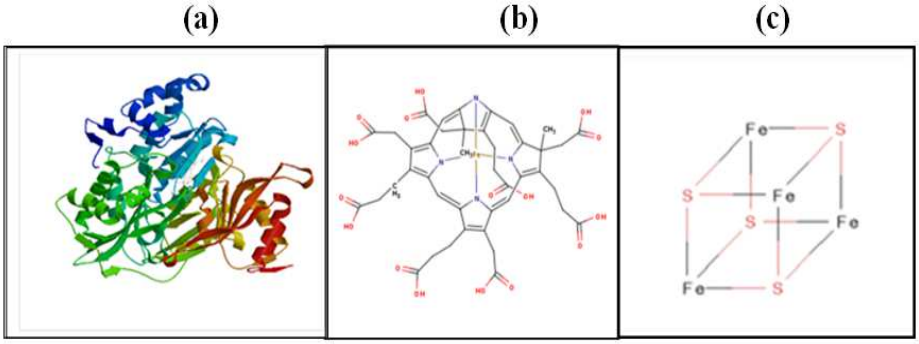


Figure 7: Predicted Protein Structure of NiR using SWISS-MODEL Program. Where: A) 3D Model of Isolated Nitrite Reductase (NiR). B) Siroheme (SRM) Ligand of Nitrite Reductase. C) SF4 Ligand of Nitrite Reductase.

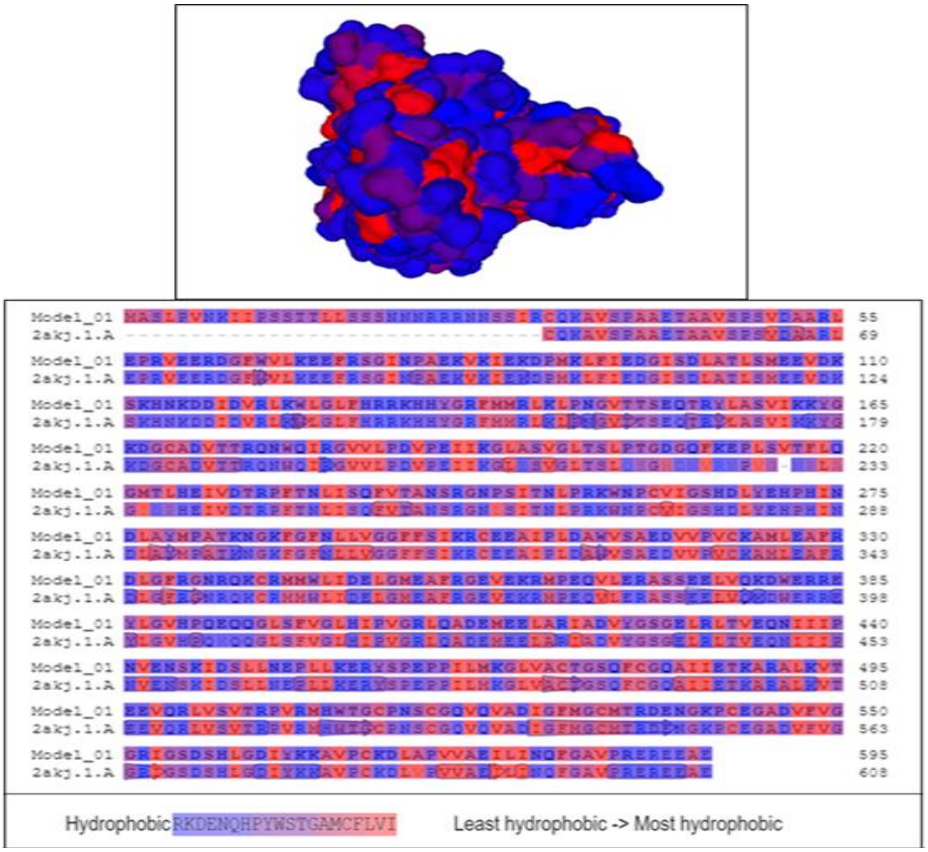


Figure 8: NiR Protein Structure and Sequence Hydrophobicity using SWISS-MODEL Program. Where the Blue Areas and Residues are the Least Hydrophobic and Red Ones are the Most Hydrophobic.

3.3 Real time PCR

To analyze the effect of potassium nitrate on the expression of nitrite reductase gene (*NiR*) in the Egyptian spinach, real time PCR was performed (Figure 9). The actin gene was used as a reference gene in the RT-PCR reactions. The expression of *NiR* transcripts and NiR protein activities has been found to be up-regulated by nitrate (**Crété et al., 1997; Wang et al., 2015**). The expression of *NiR* gene was increased until it reached the maximum at 2 hrs followed by constant-declination in expression till it reached the 24 hr-mark at which the level of expression was similar to that seen at zero time **(Orea et al., 2001)**. While, *NiR* expression reached the maximum after 8 hrs potassium nitrate induction periods in radish leaves with a 1.5- 2 fold increase and followed by rapid decrease till the 24 hrs was reached **(Wu et al., 2015)**. In tobacco plants, the nitrate treatment caused an increase in *NiR* expression by 4 folds **(Kato et al., 2004)**. It was found that amount of NO_2 uptaken by*NiR*-transformed *Arabidopsis* plants were higher than those of the wild type plants **(Takahashi et al., 2001)**.

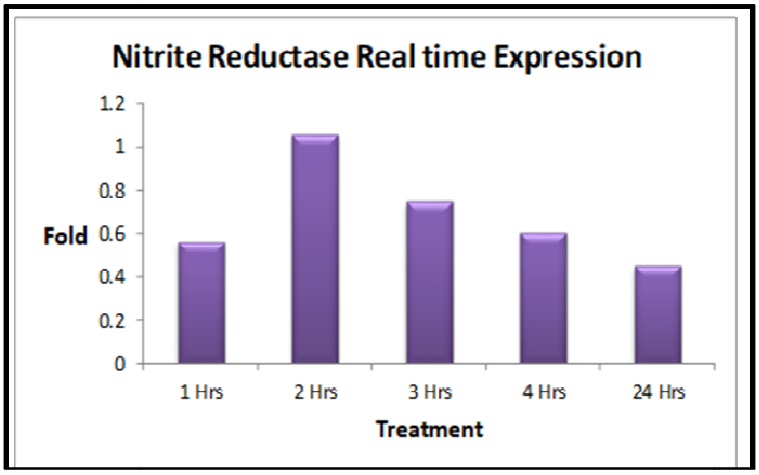


Figure 9: Nitrite Reductase Expression Chart using Real Time-PCR Reactions. It shows the Fold Change in the NiR Gene Expression in different Treatment over different Time Intervals (1, 2, 3, 4 and 24 hrs, Respectively).

3.4 Nitrite Reductase Bioassay

Using the Bradford method, the resulting absorbance and concentration of the protein with different treatments was determined using the equation y = 0.4943x obtained from the BSA standard curve. The activity of NiR protein was measured for all the different extracted protein samples. All protein samples were used with a concentration of 5.55 mg/ml/bioassay. The standard curve of different known concentrations of sodium nitrite was used in deducing the remaining concentration of nitrite in the different performed enzyme bioassays. The NiR protein was able to convert the nitrite to ammonium; therefore, the remaining nitrite concentrations were measured using Griess method that converts nitrite to azo dye which has a pink color that could be detected by spectrophotometer. The remaining nitrite concentration was then used in calculating the nitrite reductase (NiR) enzyme activity and specific enzyme activity of each teatment. The results showed that potassium nitrate treatment caused a little increase in the enzyme activity and specific enzyme activity, which was observed to increase and reach the maximium at 3 hrs (137.2 µM/min) and then the activity decreased till it reached (122.5 µM/min) at 24 hrs (Figure 10 A and B). The NiR enzyme fell behind the *NiR* mRNA. After tobacco nitrate treatment, the NiR activities were increased by 2 folds **(Kyaing et al., 2012)**.

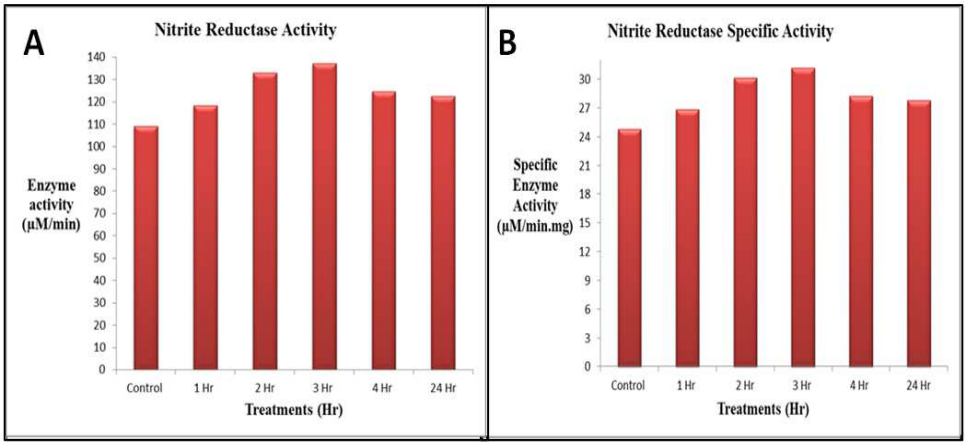


Figure 10: Effect of Potassium Nitrate on the Nitrite Reductase Activity (a) and Nitrite Reductase Specific Activity (b).

4. CONCLUSIONS

The results of this work revealed that the isolated Egyptian spinach nitrite reductase (*NiR*) gene length was 1788 bp. Its deduced amino acids consisted of 595 residues with a molecular weight of 66.5 kDa and its isoelectric point was 6.37. The *NiR* sequence was submitted to GenBank under accession number MH729808. It had a 96% homology with the predicted ferredoxin-nitrite reductase sequence, chloroplastic (*Spinacia oleracea*) and was highly conserved. The expression level of *NiR* was studied using RT-PCR which increased after 2 hrs of nitrate treatment (20 mM KNO_3). Also, after induction with nitrate and using $NaNO_2$ as a substrate in the NiR activity assay, the crude NiR protein was induced after 3 hrs of treatment.

Author's Contributions

RMA conceived the study. RMA and WAMH performed the study and provided the reagents and analysis tools. AMN and RMA analyzed the data and wrote the results. SA-E and GGM assisted in revising the manuscript and proofreading.

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