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Full Length Research Paper

Isolation, analysis and prokaryotic expression of nitric oxide associated factor gene-*NOA1* from potato (*Solanum tuberosum* L.)

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In this study, the cDNA of a NOA (nitric oxide associated factor) was isolated with the strategy of EST *in silico* cloning in combination with RT-PCR and 3'RACE from potato (*Solanum tuberosum* L.) for the first time (named *StNOA1*). The *StNOA1* bears a centrally positioned GTPase-binding domain. Sequence alignment and phylogenetic analysis of the deduced *StNOA1* protein with other known NOA family protein indicates that *StNOA1* is highly homologenous with NbNOA1 (NOA from *Nicotiana benthamiana*). The cDNA was cloned into prokaryotic expression vector, pET-30a (+) and expressed in *Escherichia coli* BL21 (DE3) after induction with IPTG. The recombinant protein was dissolved by 8 M urea and recovered by dialysis due to most of them were in inclusion bodies. Then the recovered recombinant protein was purified by Ni-NTA and analyzed by SDS-PAGE. The results of SDS-PAGE showed that the *StNOA1* was successfully expressed with the pET prokaryotic expression system and purified. The present study is the basis for further elucidating the biochemical characteristics of *StNOA1* and is very significant for elucidating the nature of plant NOA and its action mechanisms in endogenous NO synthesis in plant species.

Key words: NOS, ***Solanum tuberosum*** L., ***AtNOA1***.

INTRODUCTION

Recently, nitric oxide (NO) has been suggested to be an important signaling molecule in plants (Neill et al., 2003; Wendehenne et al., 2004; Crawford and Guo, 2005; Zhao et al., 2007). It has been shown to affect growth and development of plant tissue (Durner and Klessig, 1999), induce seed germination in stead of red light (Beligni and Lamattina, 2000), affect plant maturation and senescence (Guo and Crawford, 2005), mediate abscisic acid (ABA), induced stomatal closure and play a role in the light mediated greening (Zhang et al., 2006).

Furthermore, NO has been implicated to be involved in drought stress, salt stress and heat stress, disease resistance and apoptosis (Delledonne et al., 1998; Durner and Klessig, 1999; Mata and Lamattina, 2001; Zhao et al., 2004; Song et al., 2006). Owing to the essential role of NO in plant signaling network, its endogenous source has become the hot research focus. Increasing studies indicated that NO synthesis in plants mainly includes both nitric oxide synthase (NOS) and nitrite reductase (NR)-dependent pathways (Cueto et al., 1996; Delledonne et al., 1998; Foissner et al., 2000; Bright et al., 2006; Yamasaki et al., 1999; Yamasaki and Sakihama, 2000; Rokel et al., 2002). The Arabidopsis (***Arabidopsis thaliana***)

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Abbreviations: NOA, Nitric oxide associated factor; **NOS,** nitric oxide synthase; **NR,** nitrite reductase; **EST,** expression sequence tag; **cDNA,** complementary DNA; **RACE,** rapid amplification of cDNA ends; **RT-PCR,** reverse transcriptase-polymerase chain reaction; **StNOA1,** NOA from ***Solanum tuberosum*** L.; **NbNOA1,** NOA from ***Nicotiana benthamiana***; ***AtNOS1,*** NOS gene from ***Arabidopsis thaliana***; **OsNOA,** NOA from ***Oryza sativa;*** **VvNOA,** NOA from ***Vitis vinifera;*** **ZmNOA,** NOA from ***Zea may***; **IPTG,** isopropyl thiogalactoside; **SDS-PAGE,** sodium dodesyl sulfate-polyacrylamide gel electrophoresis; **GTP,** guanidine 5'-triphosphate; **GTPase,** guanidine 5'-triphosphatase.

Table 1. Sequence of primers used in this study.

Primer	Sequence
P_{3R}	5'-AAGCAGTGGTAACAACGCAGAGTAC d(T)$_{30}$N(AGC)(AGCT)
Stnoa1	5'-ATATCCTTCTTGTTCGAGCTTCC-3'
Stnoa2	5'-TTCGAGCTTCCTTTCACAATTAAC-3'
Stnoa3	5'-TGACCAGGAGACATACGATTTGA-3'
Pnup	5'- AAGCAGTGGTAACAACGCAGAGT-3'
Stnoa-flf	5' -CAATATCCTTCTTGTTCGAGC-3'
Stnoa-flr	5' -CCCGTATATACCTGTTGTAGCA-3'
Stnoa5	5' -GCGGTACC ATGGCGCCTAAACTCCTAGCTC-3' ***kpn1***
Stnoa6	5'-GCGGATCC TCAGAAAAACCATTTGGGTCT-3' ***BamH1***

AtNOS1 gene that was suggested to encode a protein with sequence similarity to a protein that is involved in NO synthesis in the snail ***Helix pomatia*** has been isolated (Guo et al., 2003). And its counterpart homozygous mutant line, ***AtNOS1***, was obtained by inserting T-DNA in the first exon of ***NOS1*** gene (Guo et al., 2003). ***In vivo*** NOS activity was suppressed by 75% in ***AtNOS1*** mutant plants (Guo et al., 2003). However, recent findings demonstrated that recombinant ***AtNOS1*** protein showed no NOS activity ***in vitro*** (Zemojtel et al., 2006), suggesting that ***AtNOS1*** is involved in NO biosynthesis and accumulation in either indirect or regulatory pathways. In light of these findings, it has been suggested that ***AtNOS1*** be renamed ***AtNOA1*** (Crawford et al., 2006). In spite of detection of NOS activity in several plant species, however, there is no molecular and genetic information on NOS genes in crop species.

Potato (***Solanum tuberosum*** L.) is one of the important food crops with high nutritional value and yield potential. It provides approximately half of the world's annual production of all roots and tubers, making it largest non-cereal food crop. It is a part of the diet of half a billion people in developing countries (FAO/CIP, 1995). It is continuously threatened by many abiotic and biotic stresses (Li et al., 2007).

In the present study, a potato ***NOA*** gene was cloned, expressed and purified. Sequence homology analyses and phylogenetic analysis were made. Because it was the first ***NOA*** gene isolated from potato (***S. tuberosum*** L.), it was designated as ***StNOA1***. This was the basic study for elucidating ***StNOA1*** biochemical characteristics and functions in plant development and defense responses and applying it in crop species molecular breeding ultimately.

MATERIALS AND METHODS

Plant material and main reagents

Potato (***S. tuberosum*** L. ***cv.*** Daxiyang) leaf and flower were used for RNA extraction. Trizol RNA Extract Kit was purchased from Invitrogen (USA). Ex-***Taq*** DNA Polymerase was purchased from Takara. High fidelity ***Pfu*** DNA Polymerase, ***Taq*** DNA Polymerase, DNA Gel Extraction and Purification Kit and Plasmid Extraction Kit were purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (China). Restriction enzymes were purchased from NEB (New England Biolabs). T4 DNA ligase was purchased from Promega. Reverse transcriptase and 1 kb DNA ladder were purchased from Ferment. Protein molecular mass standard was from Shanghai Biocolor Bioscience and Technology Company (China). Plasmid pET-30b (+) and ***Escherichia col.*** BL21(DE3) were purchased from Novangen. The Ni-NTA Superflow was from Amersham (Sweden).

StNOA1 5' cDNA end sequence obtaining and 3' RACE primer designing

Using ***A. thaliana NOA1*** cDNA sequence as a querying probe to search against ***S. tuberosum*** EST database in GenBank, the cDNA 5' end sequence was obtained by EST merging. The 3' RACE primers (***Stnoa1***, Stnoa2, Stnoa3, Stnoa5 and Stnoa-flf) was designed according to ***StNOA1*** 5' end sequence (Table 1).

Isolation of *StNOA1* full length cDNA by 3' EACE

For RT-PCR, total RNA was extracted from 100 mg potato fresh leaf and flower mixed samples using Trizol reagent according to manufacture's protocol. Total RNA was extracted, ethanol precipitated and re-suspended in 30 uL of RNase free sterile distilled water. First strand of cDNA was generated from 5 ug of RNA with the Superscript Reverse Transcriptase using RT primer P_{3R} (Table 1). The ***StNOA1*** 3' cDNA end was obtained by cassette PCR with primer pairs (***Stnoa1***; Stnoa2 and Pnup; Stnoa3 and Pnup) (Table 1). Subsequently the ***StNOA1*** 3' cDNA was ligated into Puc-T vector and sequenced. The gene specific primers (Stnoa-flr and Stnoa6) were designed according to the sequence of ***StNOA1*** 3' cDNA end sequence. The ***StNOA1*** cDNA full length was amplified by cassette PCR with gene specific primer pairs (Stnoa-flf and Stnoa-flr; Stnoa5 and Stnoa6) (Table 1). For 3' RACE, PCR were performed under the following conditions: 3 min at 94°C for full denaturalization, 30 s at 94°C, 30 s at 55°C, 1 min at 72°C for 30 circles of amplification and 10 min at 72°C for additional extension. For full length cDNA isolation, PCR procedure was as follows: 3 min at 94°C for full denaturalization, 30 s at 94°C, 30 s at 55°C, 2 min at 72°C for 30 circles of amplification and 10 min at 72°C for additional extension.

Sequence homology analyses and phylogenetic analysis

Sequence homology analyses were carried out with DNAMAN

software and BLAST system. Multiple protein sequence alignment was performed using the 561 amino acid domain of ***StNOA1***. Polygenetic analysis was performed by DNAMAN software. The accession numbers of the genes used in the study are as follows: ***Oryza sativa NOA*** (EAY84101CM000127.1), ***Vitis vinifera NOA***: (CAO42714CU459268.1); ***A. thaliana NOA*** (NP_850666NM_180335.1), ***Nicotiana benthamiana NOA*** (BAF93184AB303300.1), ***Zea may NOA*** (ACN26917BT062220.1) and YqeH (NC_002570.2)

Construction of prokaryotic expression vector

RT-PCR products of the ***StNOA1*** gene were gel purified and subcloned into pBluescript and SK(+) vector, designated as PBSK-***StNOA1***. The sequenced recombinant PBSK-***StNOA1*** plasmid was digested by BamH1/Kpn1 and ligated into pET-30b (+) expression vector. The recombinant plasmid was verified with PCR and restriction enzyme digestion. The correct recombinant plasmid was designated as pET-***StNOA1*** and transformed into competent cells of the ***E. coli*** strain BL21 (DE3).

Expression and purification of recombinant protein

The ***E. coli*** strain BL21 (DE3) containing correct plasmid pET-***StNOA1*** was cultured in liquid LB containing Kanamycin (50 U ml^{-1}) at 37°C with 250 rpm, when the OD_{600} reached 0.6, IPTG was added to a final concentration of 0.2 mM and the cells were cultured for another 4 h at 37°C with 300 rpm. Then the culture was centrifuged at 5000×g for 10 min at 4°C and the cells were suspended by buffer: (50 mM NaCl, 20 mM Tris-HCl, pH 8.0). The suspended cells were centrifuged again at 5000×g for 10 min at 4°C. The cells were re-suspended by the same buffer and were broken up by ultrasonic (Utrachallprozessor UP200s, 45% output power, 0.4 cycle) and then centrifuged at 12000×g for 30 min at 4°C. The inclusion bodies were dissolved by 8 mM urea and recovered by using dialysis method and purified by Ni-NTA according to the manual. The Ni-NTA spin column was pre-equilibrated by buffer: (300 mmol/ l^{-1} NaCl, 20 mmol/ l^{-1} Tris-HCl, pH 8.0). The recombinant protein was eluted using 300 mM imidazole. The eluates were analyzed by 12% sodium dodesyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

Isolation of *StNOA1* gene

The cDNA 5' end sequence of ***StNOA1*** was obtained by EST ***in silico*** cloning. The 3' RACE forward primers (***Stnoa1***, Stnoa2 and Stnoa3) were designed according to ***StNOA1*** 5' end sequence (Table 1). The 3' end sequence was obtained by 3'RACE and the gene specific primers were designed according to the 3' sequence. The ***StNOA1*** gene was successfully amplified by gene specific primer pairs (Stnoa-flf, Stnoa-flr; Stnoa-5 and Stnoa-6) (Table 1) and identified by 1% agarose gel electrophoresis (Figure 1).

Sequence alignment and phylogenetic analysis

The deduced protein of ***StNOA1*** contains 561 amino acid residues (Figure 2). Sequence alignment between ***StNOA1*** and its homologs revealed that ***StNOA1*** shared 89.44% identity with NbNOA1 (NOA from ***N. benthamiana***), 63.56% with ***AtNOA1*** (NOA from ***A. thaliana***), 63.62% with OsNOA (NOA from ***Oryza sativa***), 70.02% with VvNOA (NOA from ***V. vinifera***) and 44.79% with ZmNOA (NOA from ***Zea may***) (Figure 2). The phylogenetic analysis of ***StNOA1*** with the other NOA family proteins indicates that ***StNOA1***, NbNOA and VvNOA are in the same clade (Figure 3).

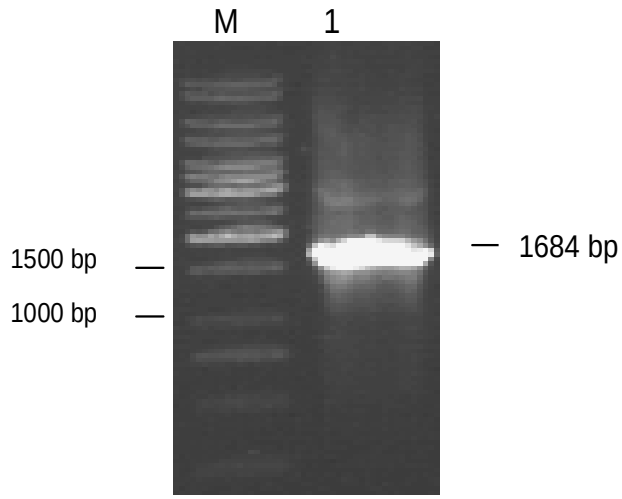

Figure 1. PCR products of StNOA1 gene. M, marker, 1 and PCR products.

Recombinant plasmid pET- 30-*StNOA1* construction

The PCR products of the ***StNOA1*** were purified and subcloned into pBluescript, SK (+) vector was sequenced and then cloned into the pET-30a (+) vector (Figure 5). The recombinant plasmid was verified by PCR and restriction enzyme digestion (Figure 6). The results of PCR and restriction enzyme digestion showed that the pET-***StNOA1*** was successfully constructed.

Expression of recombinant protein in *E. coli*

The recombinant protein was obtained by IPTG induction and the molecular mass of the recombinant ***StNOA1*** was 60 kD which was consistent with what was deduced by software. But the recombinant protein was mainly in inclusion bodies (Figure 6).

Recovering and purification of the recombinant protein

The recombinant protein was successfully recovered by using dialysis method and was affinity-purified by Ni-NTA eluted with 300 mmol/l immidazole (Figure 7).

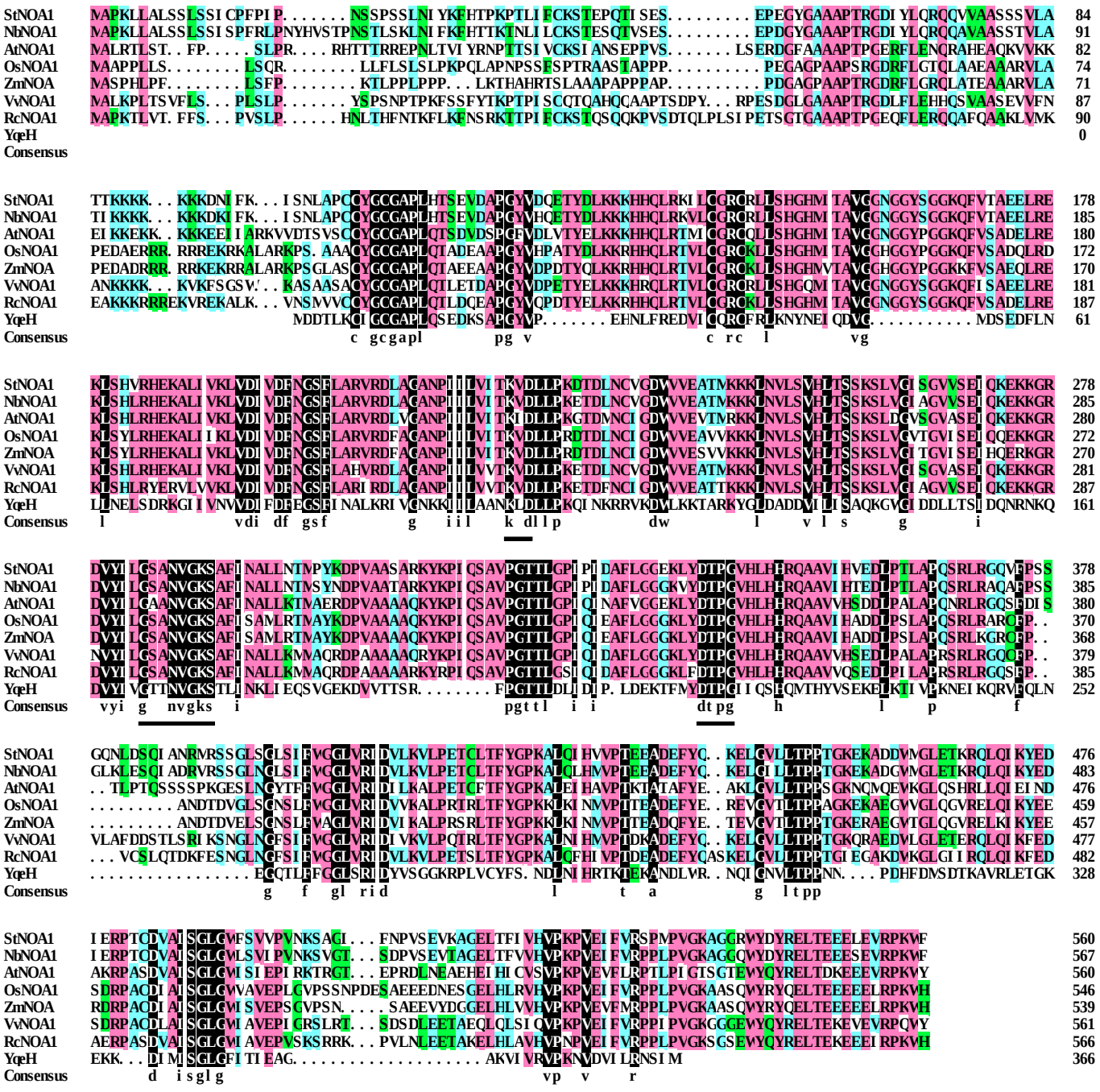

Figure 2. Alignment of the deduced amino acid sequence from StNOA1 and other NOA cDNAs. Black lines show the crucial GTPase-specific motifs of StNOA1 and its orthologous proteins: [NT]KxD (the GTP specificity motif), GxxxxGKS (Walker A), DxxG (Walker B). Abbreviations: NbNOA1, Nicotiana benthamiana (AB303300.1); AtNOA1, Arabidopsis thaliana (NM_180335.1); OsNOA1, Oryza sativa (CM000127.1); ZmNOA, Zea may (BT062220.1); VvNOA, Vitis vinifera (CU459268.1); RcNOA1, Ricinus communis (EQ973773.1)and YqeH (NC_002570.2).

DISCUSSION

Nitric oxide has emerged as a central signaling molecule in plants (Neill et al., 2003; Wendehenne et al., 2004; Crawford and Guo, 2005; Zhao et al., 2007). However, the search for a plant NO synthase (NOS) enzyme has been only at the beginning. In spite of detection of nitric oxide synthase (NOS) activity in several plant species, however, little molecular and genetic information on NOA genes has been investigated in crop species. In the present study, a ***StNOA1*** was isolated from potato and expressed it in prokaryotic cells.

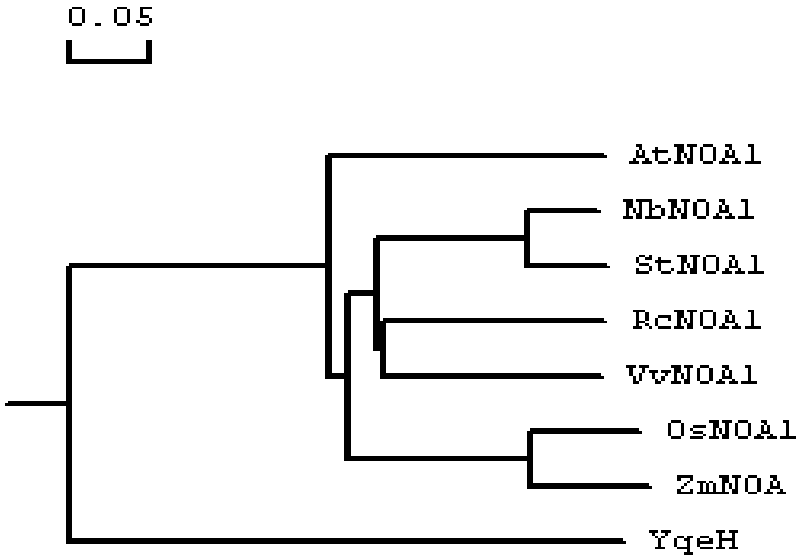

Figure 3. Phylogram of StNOA1 and other plant NOA proteins. Abbreviations: NbNOA1, Nicotiana benthamiana (AB303300.1), AtNOA1, Arabidopsis thaliana (NM_180335.1), OsNOA1, Oryza sativa (CM000127.1), ZmNOA, Zea may (BT062220.1), VvNOA, Vitis vinifera (CU459268.1), RcNOA1, Ricinus communis (EQ973773.1)and YqeH (NC_002570.2).

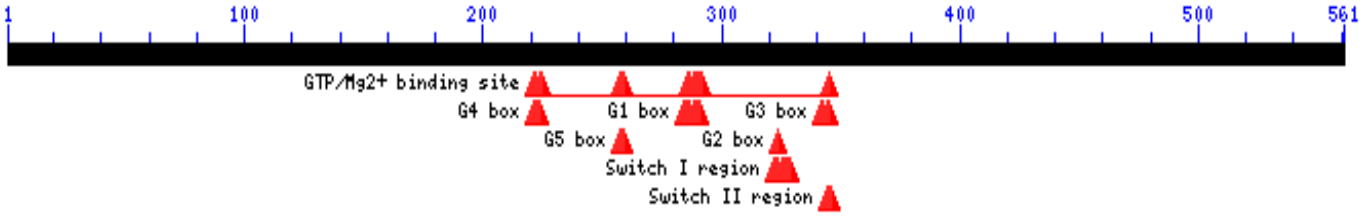

Figure 4. Putative conserved domains of deduced ***StNOA1***.

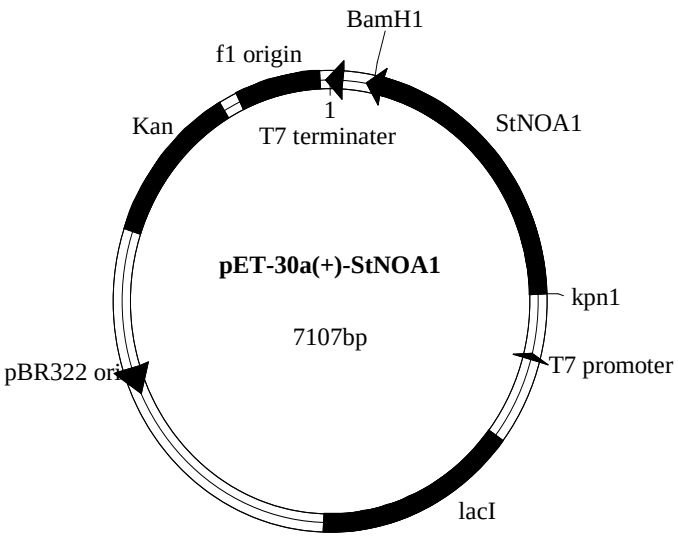

Figure 5. Prokaryotic expression vector (pET-StNOA1) construction.

EST (expression sequence tag) is the fragment of mRNA sequence after gene transcription. A large number of ESTs sequence have been obtained in the main crops and model plants. The full length cDNA information could be obtained by analysis and alignment of reported ESTs. On the basis of the alignment of cDNA information, the full length cDNA could be quickly and efficiently isolated by RACE in combination with PCR. In the present study, ***StNOA1*** full length cDNA was isolated from potato crops with the strategy of EST ***in silico*** cloning in combination with RT-PCR and 3'RACE.

The phylogenetic analysis of ***StNOA1*** with the other NOA family proteins indicates that ***StNOA1*** and NbNOA are in the same clade, they are from ***Solanaceae***. OsNOA and ZmNOA are in the same clade, which are from ***Gramineae*** (Figure 3). ***AtNOA1***as well as ***NbNOA1*** have been identified as putative regulators of NOS activity and participates in regulating endogenous NO production in plants (Guo et al., 2003; Zhao et al., 2007; Kato et al., 2008).

AtNOA1 has been recently reported to be a member of

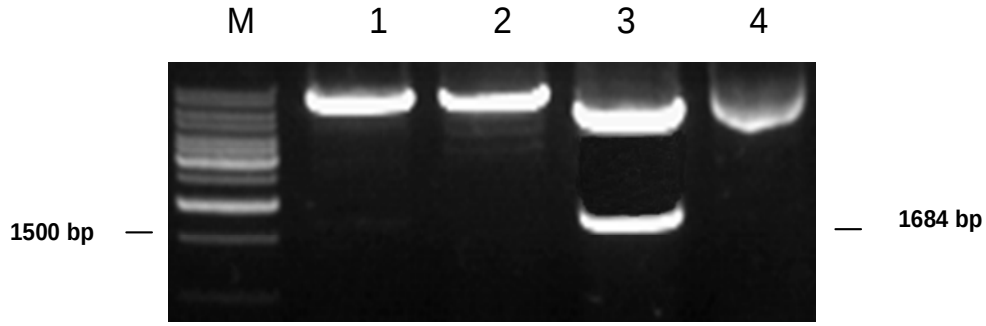

Figure 6. Identification of the recombinant plasmids by restriction enzyme digestion. **M,** marker; **1,** pET-30-***StNOA1*** digested with ***Kpn***1; **2,** pET-***StNOA1*** digested with ***BamH***1; **3,** pET- ***StNOA1*** digested with ***Kpn***1 and ***BamH***1; **4,** pET-***StNOA1***.

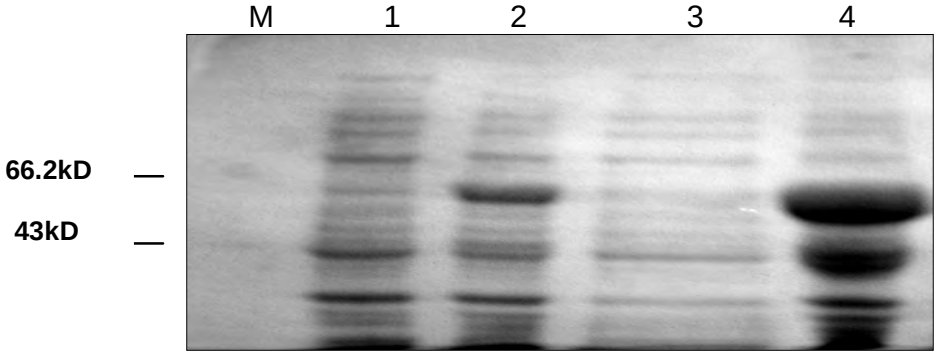

Figure 7. SDS-PAGE analysis of recombinant ***StNOA1*** protein expressed in ***E. coli.*** **M,** standard protein molecule weight marker; **1,** crude cell extracts before induction; **2,** crude cell extracts after induction; **3,** supernatants from induced ***E. coli***; **4,** pellets from induced ***E. coli***.

the circularly permuted GTPase family (cGTPase) and ***AtNOA1*** specifically binds GTP and hydrolyzes it (Moreau et al., 2008). However, complementation experiments of ***Atnoa1*** mutant plants with different constructs of ***AtNOA1*** show that GTP hydrolysis is necessary but not sufficient for the physiological function of ***AtNOA1***. Mutant ***AtNOA1*** lacking the C-terminal domain, although retaining GTPase activity, failed to complement ***Atnoa1*** (Moreau et al., 2008), suggesting the C-terminal domain may play a crucial role in plant endogenous NO production. The deduced ***StNOA1*** also bears a centrally positioned GTPase-binding domain as well as ***AtNOA1*** (Figures 2 and 4). The results suggest the functional correlation between ***StNOA1*** and ***AtNOA1*** in affecting plant growth, development and responses to stresses and pathologies by regulating endogenous NO production.

The ***StNOA1*** was expressed in prokaryotic cells. The molecular mass of the recombinant ***StNOA1*** was 60 kD, which was in agreement with what was expected. The ***StNOA1*** gene was expressed with the pET prokaryotic expression system, which was under the control of T7 promoter. The pET expression system had His-Tag sequences, which facilitated the subsequent purification of the recombinant protein. The ***StNOA1*** protein was efficiently expressed, but most of the recombinant protein was in the inclusion bodies, which might be because of the strong induction by IPTG resulted in the depression of protein synthesis in host cells.

The present study supplied a basis for elucidating ***StNOA1*** biochemical characteristics and is very significant for exploring the nature of NOA and its action mechanisms in endogenous NO synthesis and its functions in plant growth, development and defense responses and applying it in crop species molecular breeding ultimately (Figure 8).

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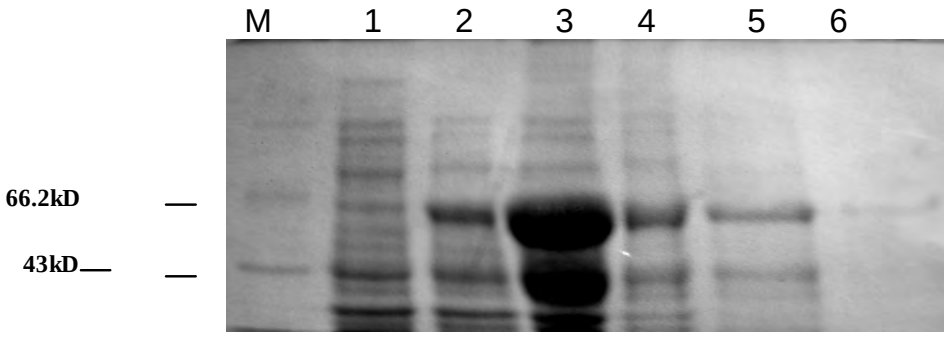

Figure 8. SDS-PAGE analysis of recombinant and purified ***StNOA1*** protein expressed in ***E. coli.*** **M,** standard protein molecule weight marker; **1,** crude cell extracts before induction; **2,** crude cell extracts after induction; **3,** pellets from induced ***E. coli***; **4,** dissolved inclusion body before dialysis; **5,** dissolved inclusion body after dialysis; **6,** affinity-purified recombinant ***StNOA1*** eluted by 300 mmol/l immidazole.

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