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马铃薯 *NOA* 基因的克隆及序列分析

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摘　要:以马铃薯(*Solanum tuberosum*)为实验材料,利用电子克隆和 RACE 技术,从马铃薯中克隆出 *NOA*(nitric oxide associated factor)基因,命名为 *StNOA1*,测序结果表明,其 cDNA 序列长度为 1 929 bp,此片段包含一个长为 1 632 bp 的完整编码框。氨基酸序列比对分析表明,StNOA1 与烟草(*Nicotiana benthamiana*),葡萄(*Vitis vinifera*),蓖麻(*Ricinus communis*),水稻(*Oryza sativa*),玉米(*Zea mays*)以及拟南芥(*Arabidopsis thaliana*)均有很高的同源性(89.44%～63.56%)。同 AtNOA1 一样,StNOA1 也具有保守的 GTP 结合区。从结构分析结果推测,StNOA1 和 AtNOA1 在功能上有一定的相关性,其也可能通过调节内源 NO 的释放参与到植物生长、发育、抗逆等过程中。

关键词:马铃薯;分子克隆;GTPase

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Molecular Cloning and Analysis of a *NOA* Gene in *Solanum tuberosum*

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Abstract: The *NOA* (nitric oxide associated factor) gene encodes a NOA which participates in nitric oxide synthase (NOS) dependent NO synthesis. In this study, the cDNA of *StNOA1* was cloned by in silico cloning and combination with RACE from potato (*Solanum tuberosum*). The full length of *StNOA1* cDNA had 1 929 bp with an open reading frame of 1 632 bp. Phylogenetic analysis revealed that the StNOA1 amino acid sequence shared high identity (89.44%～63.56%) with the NOA from *Nicotiana benthamiana*, *Vitis vinifera*, *Ricinus communis*, *Oryza sativa*, *Zea mays* and *Arabidopsis thaliana*. The StNOA1 bears a centrally positioned GTPase-binding domain as well as AtNOA1. Its sequence conservation and structure similarity with AtNOA1 implied the functional correlation between StNOA1 and AtNOA1 in plant growth, development, and responses to stresses and pathologies by regulating endogenous NO production.

Key words: *Solanum tuberosum*; molecular cloning; GTPase

Nitric oxide (NO) has been suggested to be an important signaling molecule in plants[1-4]. NO has been shown to affect growth and development of plant tissue[5], induce seed germination in stead of red light[6], affect plant maturation and senescence[7], mediate abscisic acid (ABA) induced stomatal closure, and

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play a role in the light mediated greening[8]. Furthermore, NO has been implicated to be involved in drought, salt, and heat stresses, disease resistance and apoptosis[9-12]. Owing to the essential role of NO in plant signaling network, its endogenous source has become very important. Increasing studies indicated that NO synthesis in plants mainly includes both nitric oxide synthase (NOS) and nitrite reductase (NR)-dependent pathways. *AtNOA*1 has been proven to participate in endogenous NO production in the process of hormonal signaling and in defense responses to pathogen attack and salt stress.

In present study, a full length cDNA which was homologous with *AtNOA*1 cDNA was obtained by *in silico* cloning and 3′-RACE from potato (*Solanum tuberosum*). Sequence alignments analyses and phylogenetic analysis were made. Because it was the first *NOA* gene isolated from potato, it was designated as *StNOA*1.

1 Material and methods

1.1 Plant material

Potato (*Solanum tuberosum*) was grown in greenhouse under conditions of 14 h photoperiod and 23℃.

1.2 Methods

1.2.1 General RNA extraction Potato leaves were used for RNA extraction using the Trizol reagent according to the manufacturer's instructions. RNA was precipitated by ethanol and suspended in 30 μL of RNase free sterile distilled water. General RNA sample was stored in −70℃ for following RT-PCR.

1.2.2 *StNOA*1 3′ cDNA end isolation and sequencing First strand of cDNA was generated from 5 μg of RNA with the Superscript Reverse Transcriptase using RT primer P_{3R} (Table 1). The *StNOA*1 3′ cDNA end was obtained by cassette PCR with primer pairs (Stnoa1; Stnoa2 and Pnup; Stnoa3 and Pnup) (Table 1). Subsequently the *StNOA*1 3′ cDNA fragment was ligated into Puc-T vector and sequenced. PCR were performed under the following conditions: 3 min at 94℃ for full denaturalization, 3 min at 94℃, 30 min at 55℃, 1 min at 72℃ for 30 cycles of amplification, and 10 min at 72℃ for additional extension.

Table 1 Sequence of primers used in this study

Primer name	Sequence(5′−3′)
P_{3R}	AAGCAGTGGTAACAACGCAGAGTACTTTTTTTTT-TTTTTTTTTTTTTTTTTTTT(AGC)(AGCT)
Stnoa1	ATATCCTTCTTGTTCGAGCTTCC
Stnoa2	TTCGAGCTTCCTTTCACAATTAAC
Stnoa3	TGACCAGGAGACATACGATTTGA
Pnup	AAGCAGTGGTAACAACGCAGAGT
Stnoa-flf	CAATATCCTTCTTGTTCGAGC
Stnoa-flr	CCCGTATATACCTGTTGTAGCA
Stnoa5	GCGGTACCATGGCGCCTAAACTCCTAGCTC *Kpn* I
Stnoa6	GCGGATCCTCAGAAAAACCATTTGGGTCT *Bam*H I

1.2.3 *StNOA*1 full length cDNA isolation, sub-cloning, and sequencing The *StNOA*1 full length cDNA was amplified by cassette PCR with gene specific primer pairs (Stnoa-flf and Stnoa-flr; Stnoa5 and Stnoa6) (Table 1). The gene specific primers were designed according to EST alignment sequence and 3′ end sequenced results. PCR were performed under the following conditions: 3 min at 94℃ for full denaturalization, 3 min at 94℃, 30 min at 55℃, 2 min at 72℃ for 30 cycles of amplification, and 10 min at 72℃ for additional extension. RT-PCR products of the *StNOA*1 gene were purified and sub-cloned into pBluescript Ⅱ SK(+) vector. The recombinant plasmid was transformed into competent cells of the *E. coli* strain DH5α and cultured in solid LB (100 mg/L) under 37℃ overnight. The white bacterium spot was selected for PCR verification. Then the recombinant plasmid was verified with restriction enzyme digestion. The bacterium clone containing correct recombinant plasmid was sent to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China) for sequencing.

1.2.4 Sequence comparison between *StNOA*1 and other plant NOA The sequence of *StNOA*1 was used for BLAST in GenBank (www.ncbi.gov.). The DNAMAN software was used for prediction of amino acid sequence and phylogenetic analysis between StNOA1 and other plant NOA. The accession numbers of the genes used in the study are as follows: *Oryza sativa NOA* (EAY84101), *Vitis*

vinifera NOA (CAO42714); *Arabidopsis thaliana NOA* (NP_850666), *Nicotiana benthamiana NOA* (BAF93184), *Zea mays NOA* (ACN26917), *Ricinus communis NOA* (EQ973773).

2 Results and analysis

2.1 General RNA extraction and *StNOA1* 3′ cDNA isolation

Potato general RNA was successfully extracted by Trizol reagent and identified by 1% agarose gel electrophoresis (Fig. 1). The 3′ end was successfully amplified by cassette PCR. The amplified 3′ end length was about 1 500 bp(Fig. 2).

2.2 Cloning of *StNOA1* cDNA

A 1 632 bp cDNA containing *Kpn* I and *Bam*H I enzyme cut sites was obtained by cassette PCR with gene specific primer pairs (Stnoa-flf and Stnoa-flr; Stnoa5 and Stnoa6) (Fig. 3). Enzyme digestion showed that *StNOA1* was successfully cloned to pBluescript II SK(+) vector, it was designated as pBSK-*StNOA1* (Fig. 4).

2.3 Sequence analysis of StNOA1 and other plant NOA

From its amino acids sequence deduced by DNAMAN software, StNOA1 also bared a GTP binding domain as well as AtNOA1(Fig. 5). Phylogenetic tree was constructed by DNAMAN software. The result indicated that StNOA1 and NbNOA1(NOA from *Nicotiana benthamiana*) were clustered into a subclass due to the high identity (89.44%). The subclass was phylogenetically near with the NOA from *Oryza sativa* and *Zea mays* NOA from *Arabidopsis thaliana* and *Ricinus communis* were clustered into the same subclass. NOA from *Vitis vinifera* was clustered into another subclass(Fig. 6).

Fig. 1 Electrophoretic pattern of potato RNA in 1% agrose gel

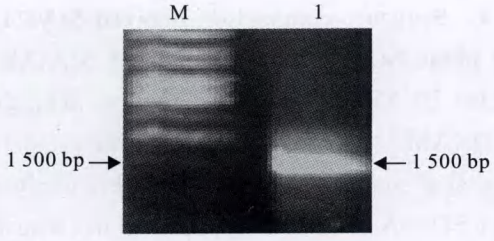

Fig. 2 *StNOA1* 3′ end amplification
M. Marker; 1. PCR products

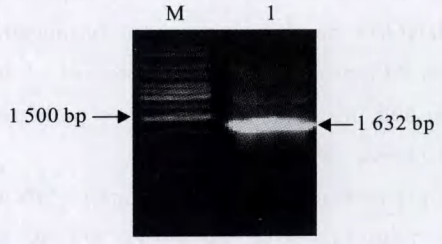

Fig. 3 PCR products of *StNOA1* gene
M. Marker; 1. PCR products

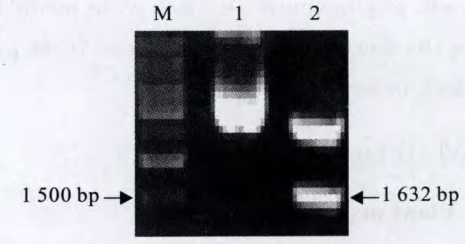

Fig. 4 Enzyme digestion of pBSK-StNOA1
M. Marker; 1. pBSK-*StNOA1*; pBSK-*StNOA1* digested with *Kpn* I and *Bam*H I

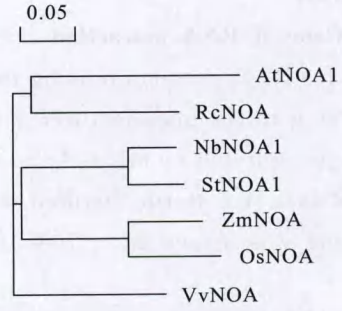

Fig. 6 Phylogenetic tree based on the amino acid sequence of NOAs from different plants
At. *Arabidopsis thaliana*; Nb. *Nicotiana benthamiana*; Os. *Oryza sativa*; St. *Solanum tuberosum*; Vv. *Vitis vinifera*; Zm. *Zea mays*; Rc. *Ricinus communis*

3 Discussion

EST (expression sequence tag) drived from mRNA. A large number of EST sequences have been obtained in the main crops and model plants. The full length cDNA information could be obtained

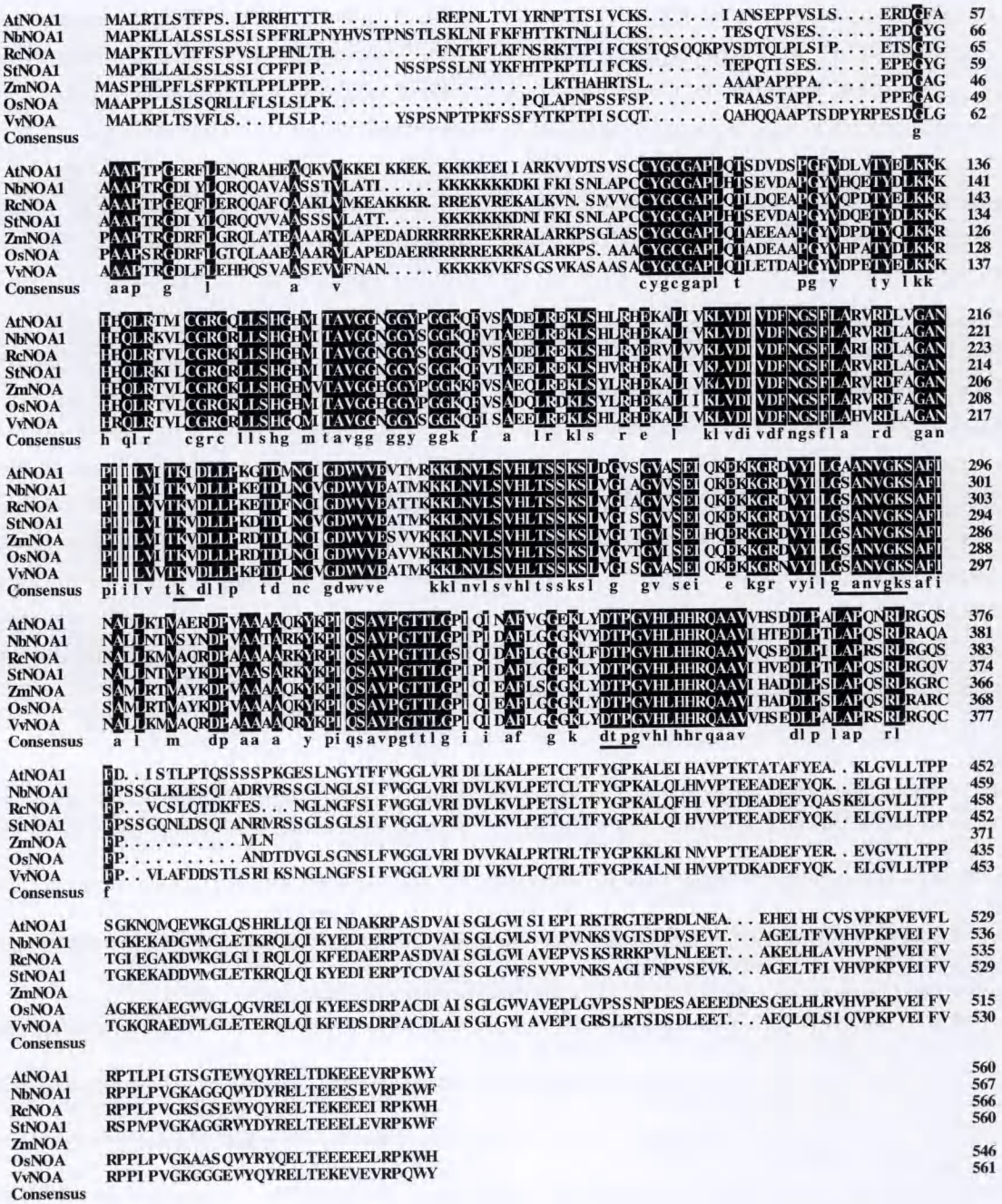

Fig. 5　Comparison of amino acid sequences between *Solanum tuberosum* NOA and other NOA

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). At. *Arabidopsis thaliana*; Nb. *Nicotiana benthamiana*; Os. *Oryza sativa*; St. *Solanum tuberosum*; Vv. *Vitis vinifera*; Zm. *Zea mays*; Rc. *Ricinus communis*

by analysis and alignment of reported ESTs. On the basis of the alignment cDNA information, the full length cDNA could be quickly and efficiently isolated by RACE in combination with PCR. In the present study, *AtNOA1* was used as the template to search potato EST sequence in GenBank. The highly homogenous EST sequences were obtained. The cDNA sequence information with complete ORF(open reading frame) was obtained. The cDNA of *StNOA1* was efficiently isolated by the RACE-PCR.

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 (Fig. 6). *AtNOA1* as well as *NbNOA1* have been identified as putative regulators of NOS activity and participates in regulating endogenous NO production in plants[4,13,11].

AtNOA1 is the first gene that was found to participate in NO synthesis in plant species. It has been recently reported to be a member of the circularly permuted GTPase family (cGTPase) and AtNOA1 specifically binds GTP and hydrolyzes it[15]. However, complementation experiments of Atnoa1 mutant plants with different constructs of AtNOA1 show that the C-terminal domain of the AtNOA1 may play a crucial role in plant endogenous NO production[15]. The deduced StNOA1 also bared a centrally positioned GTPase-binding domain as well as AtNOA1(Fig. 5). Its sequence conservation and structure similarity with AtNOA1 implied the functional correlation between StNOA1 and AtNOA1 in plant growth, development, and responses to stresses and pathologies by regulating endogenous NO production.

The present study is the basis for elucidating molecular information and biochemical characteristics of StNOA1, and is very significant for elucidating the nature of NOA and its action mechanisms in NO synthesis in plant as well as its functions in plant development and defense responses, and applying it in crop species molecular breeding ultimately.

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