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Isolation and Characterization of a Nitric Oxide-induced Gene in Sweetpotato

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A new nitric oxide-induced (NOI) gene was isolated by screening ESTs from a cDNA library of dehydration-treated fibrous roots of sweetpotato (*Ipomoea batatas*). The 720 bp cDNA fragment, *IbNOI*, was sequenced, from which a 77 amino acid residue protein was deduced. A search of the protein BLAST database identified significant similarity to other plant NOI protein sequences. Quantitative RT-PCR analysis revealed diverse expression patterns of *IbNOI* in various tissues of the intact sweetpotato plant, and in leaves exposed to different stresses. The *IbNOI* gene was highly expressed in storage roots and suspension-cultured cells. In leaf tissues, *IbNOI* showed strong expression during sodium nitroprusside (SNP)-induced NO accumulation and chemical stress treatments. Expression of *IbNOI* was also induced under various abiotic stress conditions, such as dehydration, salt, and bacterial pathogen infection. These results suggest that *IbNOI* is involved in plant responses to diverse abiotic stresses and pathogen infection through a NO-related pathway.

Key words : *Ipomoea batatas*, nitric oxide, reactive oxygen species, stress

Introduction

Nitric oxide (NO) is a gaseous free-radical and key signaling molecule in different physiological processes of animals and plants [9, 10]. In plants, NO is a relatively stable paramagnetic free-radical molecule involved in many physiological processes, where it functions as a synchronizing chemical messenger involved in cytotoxicity and programmed cell death during pathogen attack [2, 10]. NO is implicated in the response to some abiotic stresses in different plant species [1, 3]. The NO intracellular signaling cascade is activated in plant cells in response to pathogens or elicitors [4]. At the transcriptional level, a microarray study obtained from NO donor-treated Arabidopsis cells indicates that NO modulates the expression of several defense-related genes, including genes encoding pathogenesis-related (PR) and secondary metabolism-related proteins [11, 13].

Sweetpotato (*Ipomoea batatas*) is a relatively drought-resistant crop and represents one of the most important root crops grown on marginal lands. Although sweetpotato is recognized as a comparatively drought-tolerant plant, the molecular mechanisms underlying drought tolerance are not well defined. Previously, expressed sequence tags (ESTs) from a full-length enriched cDNA library prepared from dehydration-treated fibrous roots of sweetpotato were isolated and characterized [6]. Expression analysis showed that some sweetpotato antioxidant genes isolated from the EST library of dehydration-treated fibrous roots were also induced in response to various environmental stresses [7]. Therefore, the investigation of sweetpotato EST pools might yield valuable genetic information about the regulatory networks involved in stress-response processes of sweetpotato.

In this study, we isolated and characterized the NO-induced *IbNOI* gene from an EST library of dehydration-treated fibrous roots of sweetpotato. To better understand the function of the *IbNOI* gene, we analyzed its expression under various stress conditions. The results suggest that the *IbNOI* gene plays a role in responses to abiotic stress and pathogen infection in sweetpotato.

Materials and Methods

Plant materials

Sweetpotato (*Ipomoea batatas* L. Lam. cv. White Star) was obtained from Bioenergy Crop Research Center, National

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Crop Research Institute, RDA, Muan, Jeonnam, Korea. Plants were cultivated in a growth chamber in soil at 25℃ with a photoperiod of 16 hr light/8 hr dark for 50 days.

Analysis of DNA and protein sequences

Sequence identities were determined using the National Center for Biotechnology Information (NCBI) BLAST search tool (https://blast.ncbi.nlm.nih.gov/), and multiple sequence alignments were performed using the Clustal X (www.clustal.org/clustal2/) and GeneDoc programs. The deduced proteins were predicted using the ExPasy (http://www.expasy.org/tools) programs.

Stress treatment

Sweetpotato plants grown at 25℃ for 50 days were used for stress treatments. For stress treatment conditions, sweetpotato plants were incubated in a growth chamber at 25±3℃ under a photoperiod of 16 hr light/8 hr dark, and 70% relative humidity) with a light intensity of 200 μmol s^{-1} m^{-2} provided by fluorescent and metal halide lamps. For sodium nitroprusside (SNP) treatment, the third leaf from the top was removed from each plant and transferred to Petri dishes containing 50 ml of each solution at different concentrations (0, 0.1, 0.5, 1, 5, 10, and 50 mM) for 24 hr. For treatments with hydrogen peroxide (H_2O_2, 400 mM), methyl viologen (MV, 0.05 mM), cadmium (Cd, 0.5 mM), and copper (Cu, 0.5 mM), sweetpotato leaves were incubated in conical tubes containing 30 ml of each chemical solution at 25℃ for 24 hr under light conditions. Sterile water was used as a control for chemical stress treatments. For dehydration treatment, the plants were carefully pulled out, transferred onto filter papers and allowed to dry. Sweetpotato leaves were collected at 0 (untreated control), 1, 2, 4, 8, 16, and 24 hr after treatment. For treatments with NaCl, the third leaf from the top was removed from each plant and placed into conical tubes containing 30 ml of sterile water (control), or 100 mM NaCl, and then incubated at 25℃ for 24 hr. For bacterial treatment, *Pectobacterium chrysanthemi* (*Erwinia chrysanthemi*, KCTC 2569) was used according to Jang et al. [5]. *P. chrysanthemi* was grown on nutrient agar medium at 30℃. For infection with *P. chrysanthemi*, nine leaf discs (18 mm in diameter) placed in a Petri-dish (87×15 mm) were inoculated with *P. chrysanthemi* (1.3×10^4 cells per ml). Bacterial cell numbers were estimated by measuring the optical density at 600 nm. For Mock treatment, leaf discs were treated with distilled water. All treated plant materials were frozen immediately in liquid nitrogen after treatment and stored at -70℃ until further use.

Gene expression analysis

Total RNA was isolated from sweetpotato using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated extensively with RNase-free DNase I to remove any contaminating genomic DNA. For quantitative expression analysis of antioxidant genes, first strand cDNA was generated from total RNA (2 μg) using MMLV reverse transcriptase (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed in a fluorometric thermal cycler (DNA Engine Opticon 2, MJ Research, Waltham, MA, USA) using Ever Green fluorescent dye, according to the manufacturer's instructions. The inter-experimental quality control comparisons of repeated samples were assessed using CT values between the three replications. Linear data were normalized to the mean CT of α-tubulin as a reference gene. Gene-specific primers used for PCR reactions were as follows: the IbNOI primer set (5′-GCCAGCCAGAATCACCAACAAA-3′, 5′-TCCACATGCAAAATGTGGGGAT-3′) used to amplify a 180 bp product from cDNA coding for *IbNOI*; and *alpha-tubulin* gene-specific primers (5′-CAACTACCAGCCACCAACTGT-3′, 5′-CAAGATCCTCACGAGCTTCAC-3′) to amplify the tubulin gene as an internal standard.

Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA). The subsequent multiple comparisons were examined based on the least significant difference (LSD) test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 12), and statistical significance was set at $p<0.05$ and $p<0.01$.

Results

Isolation and sequence analysis of the IbNOI

To analyze the ESTs from the cDNA library of dehydration-treated sweetpotato fibrous roots [9], we searched the sequences using the NCBI database. The classification was based on the best homology match of BLASTX searches against Arabidopsis and other plant protein sequences. The novel nitric oxide-induced (NOI) gene was found among the most abundant ESTs. Its cDNA is 712 bp in length and encodes a deduced 77 amino acid residue protein (Fig. 1A).

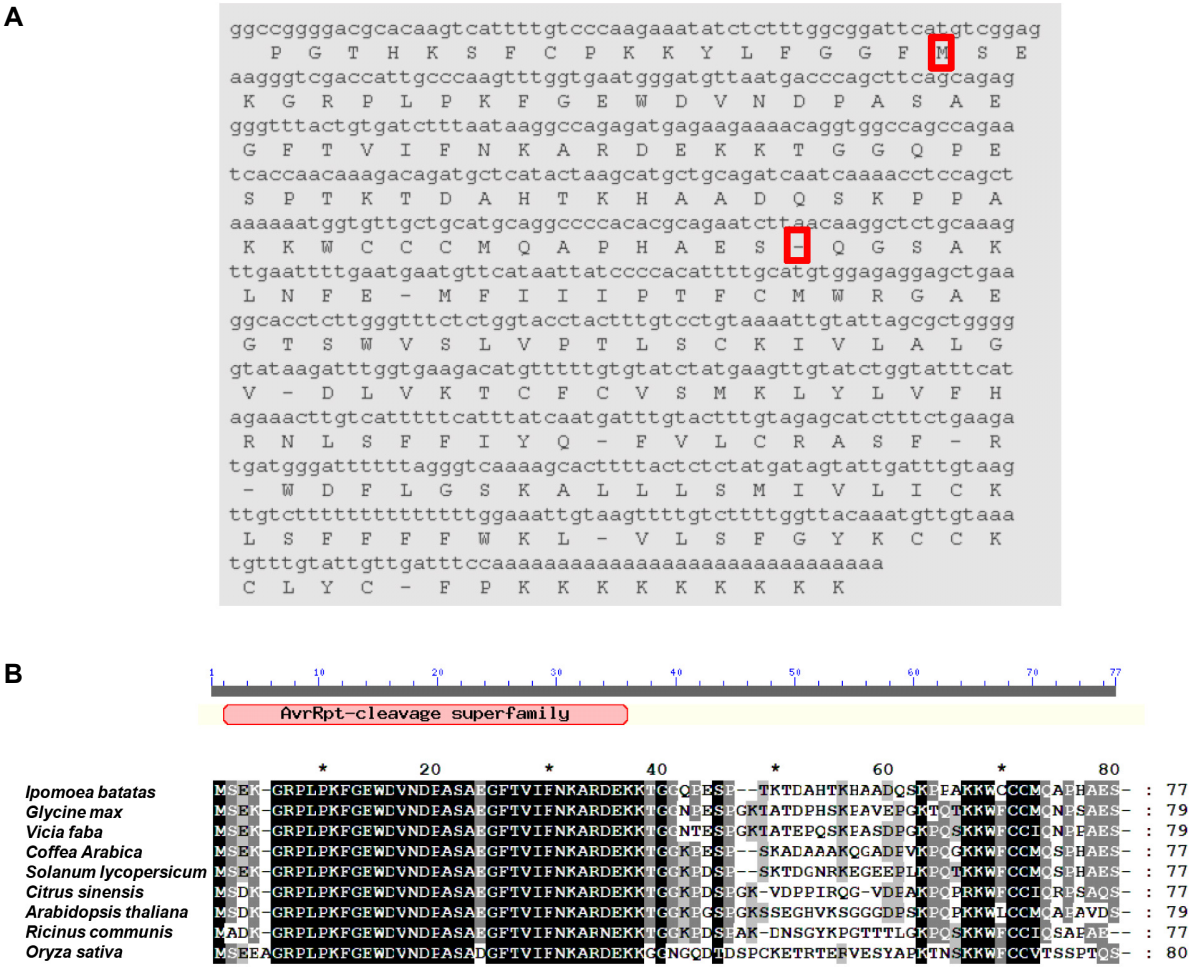

Fig. 1. Sequence analysis of the IbNOI full-length cDNA. (A) Nucleotide and deduced amino acid sequences of IbNOI cDNA. (B) Multiple alignments of the amino acid sequences of INOI proteins isolated from various plants. Conserved AvrRpt-cleavage superfamily domain was shown in red box. *Ipomoea batatas* (MK455780), *Glycine max* (XP_003532179.1), *Vicia faba* (BAJ78239.1), *Coffea Arabica* (XP_027062349.1), *Solanum lycopersicum* (XP_004244039.1), *Citrus sinensis* (XP_006474872.1), *Arabidopsis thaliana* (AAB86938.1), *Ricinus communis* (XP_002532206.1), *Oryza sativa* (BAD69095.1).

Database searches revealed that the amino acid sequence of the IbNOI protein contains a conserved, 33 amino acid sequence homologous to the AvrRpt-cleavage superfamily domain that is present in a large family of plant AvrRpt-cleavage proteins (Fig. 1B). Alignment and comparison of its amino acid sequence with those of other NOI genes isolated from various plant species showed that it shares 83% homology with coffee NOI and 79% homology with tomato NOI (Fig. 1B). Therefore, we suggest that IbNOI can be classified as a member of the plant NOI proteins, and is likely to have a similarly important role in NO-related stress responsive gene expression.

Differential expression of *IbNOI* in intact sweetpotato tissues

The expression patterns of the *IbNOI* gene were investigated in various whole plant tissues (L, leaf; S, stem; FR, fibrous root; TR, thick pigmented root; SR, storage root; SUS, suspension-cultured cells) by RT-PCR analysis (Fig. 2). The results demonstrate that there is considerable variation in the levels of *IbNOI* expression in different sweetpotato tissues. The *IbNOI* gene was strongly expressed in storage root and suspension-cultured cell conditions, whereas it was weakly expressed in leaf, stem, and fibrous root tissues.

Differential responses of sweetpotato *IbNOI* to SNP and chemical stresses

To investigate the NO-induced responses of sweetpotato *IbNOI*, gene expression changes in response to increasing concentrations of SNP in leaves of sweetpotato were ana-

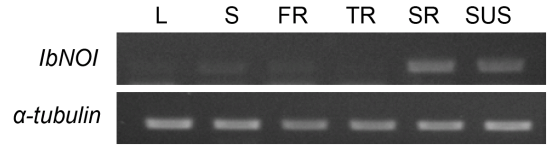

Fig. 2. Expression patterns of *IbNOI* in various intact tissues of sweetpotato. Total RNAs were extracted from the leaf (L), stem (S), fibrous root (FR), thick pigmented root (TR), and storage root (SR), and suspension-cultured cells (SUS). Alpha-tubulin was utilized as a control for equal loading. RT-PCR analyses were repeated at least three times.

lyzed by qRT-PCR. The *IbNOI* gene exhibited different expression patterns in response to increasing concentrations of SNP (Fig. 3A). The *IbNOI* gene was strongly expressed in response to 1 mM SNP. *IbNOI* expression was induced by various reactive oxygen species and heavy metal in sweetpotato leaves (Fig. 3B).

Differential expression of *IbNOI* in response to pathogen infection and abiotic stresses

The expression patterns of *IbNOI* in response to abiotic stress treatment and pathogenic infection were investigated by qRT-PCR. Following dehydration, *IbNOI* was differentially expressed in leaves (Fig. 4A). *IbNOI* expression increased strongly 4 hr after dehydration, whereas in the control conditions expression was slightly up-regulated at 16 h. In leaves, expression of the *IbNOI* gene was induced within 2 hr by treatment with 100 mM NaCl (Fig. 4B). Expression patterns of the *IbNOI* gene were also investigated in the leaves after infection with a bacterial pathogen (*P. chrysanthemi*) (Fig. 4C). *IbNOI* expression was strongly induced by bacterial infection from 8 to 20 hr, and increased under untreated control conditions after 20 hr. These results suggest that *IbNOI* responds differently to various kinds of abiotic stress and pathogen infection.

Discussion

In this study, *IbNOI* was isolated from dehydration-treated fibrous roots of sweetpotato, and the response to abiotic stress and pathogen infection was characterized. A comparison of stressed and unstressed plants revealed that the expression levels of the *IbNOI* gene increased in response to high concentration of SNP (1 mM), dehydration, NaCl, MV, Cd treatment, and bacterial pathogen infection (Fig. 3, Fig. 4). However, the expression levels of *IbNOI* did not change significantly in response to H_2O_2 and Cu treatments. These results suggest that the *IbNOI* gene is involved in responses to NO-related abiotic stresses and pathogen defense-mediated plant stresses in sweetpotato.

IbNOI was also found to be expressed differentially in various tissues of whole plants and under cell culture conditions of sweetpotato (Fig. 2). Interestingly, they showed the highest expression during growth as cell suspension cultures. Cell suspension cultures result in the exposure of cells to higher levels of oxidative stress compared with whole plants [8]. The results of the present study showed that the expression of the *IbNOI* gene increased following oxidative stress treatment, such as with MV and Cd (Fig. 3). These findings indicate that the expression of the *IbNOI*

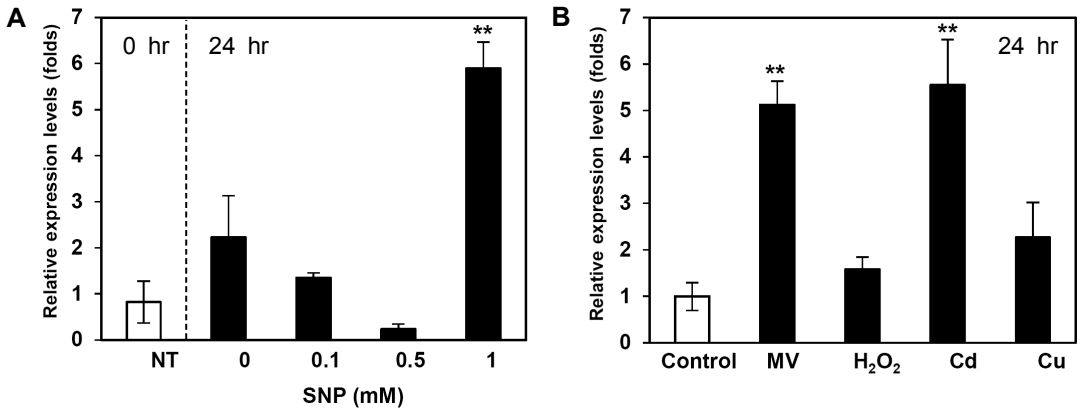

Fig. 3. Expression patterns of *IbNOI* under SNP and chemical stress conditions. (A) Expression patterns of *IbNOI* leaves in response to SNP treatment for 12 hr. (B) Expression patterns of *IbNOI* under stress-related chemical treatments (0.05 mM MV, 400 mM H_2O_2, 0.5 mM Cd, and 0.5 mM Cu) for 24 hr. Data represent the average of three replicates from two experiments, and error bars indicate SD of the means. Statistical significance of differences between the control and treatment groups was determined by one-way ANOVA with the LSD post hoc test (*p<0.05; **p<0.01).

gene might be regulated in cell suspension cultures and under stress-related chemical treatment-mediated oxidative stress conditions.

Interestingly, *IbNOI* gene was markedly expressed in response to bacterial infection, and this gene expression was also lately increased in mock treatment by wounding effects (Fig. 4). In the previously study, expression patterns of 10 peroxidase genes were investigated in sweetpotato during *P. chrysanthemi* infection [5]. The peroxidase genes also showed the induced gene expression in mock treatment was well matched with the induction by wounding in their previous study [13]. Strong induction by wounding was shown in sweetpotato peroxidase gene swpa4 which also showed strong response to pathogen infection similarly with *IbNOI*. These results suggested that *IbNOI* gene contribute to a defense mechanism against pathogen attack.

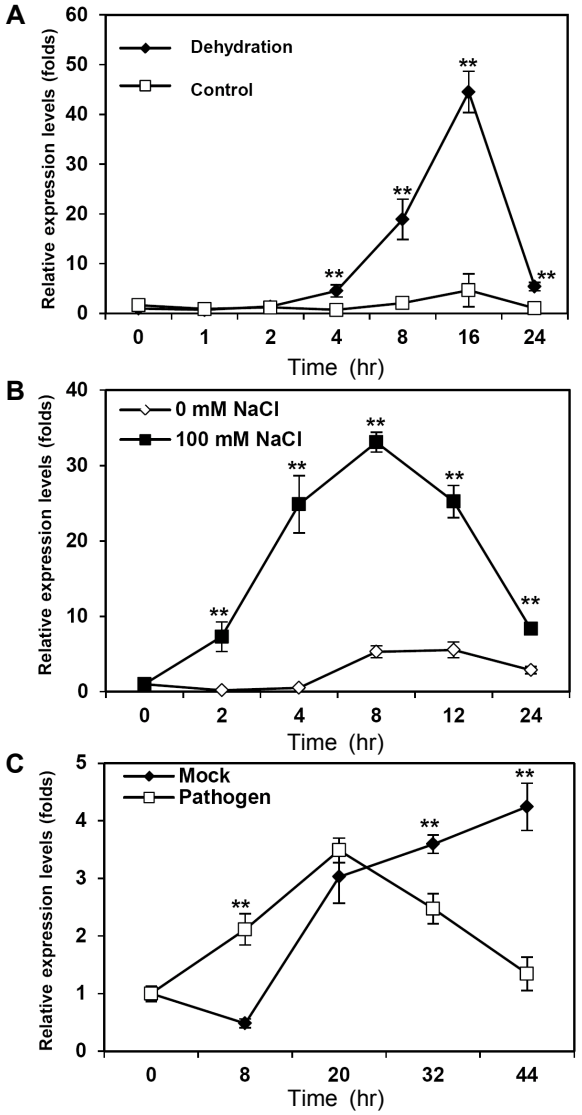

Fig. 4. Expression patterns of *IbNOI* under different abiotic stress and pathogen infection conditions. (A) Expression patterns of *IbNOI* in leaves under dehydrated conditions for 24 hr. (B) Expression patterns of *IbNOI* in leaves treated with 100 mM NaCl for 24 hr. (C) Expression patterns of *IbNOI* following infection of sweetpotato leaves with a bacterial pathogen (*P. chrysanthemi*). The mock involved treatment with 10 mM $MgCl_2$. Data represent the average of three replicates from each of two experiments, and error bars indicate SD of the means. Statistical significance of differences between the control and treatment groups was determined by one-way ANOVA with the LSD post hoc test (*p<0.05; ** p<0.01).

The data presented here suggest that *IbNOI* might have roles in NO-related abiotic stress tolerance and pathogenic resistance. Further investigation will be required to elucidate the exact role of the *IbNOI* gene in the regulation of the defense signal pathway in sweetpotato under stress conditions. For analyses of its role in the stress tolerance mechanism in plants, transgenic sweetpotato expressing the *IbNOI* gene will be generated. We expect that these studies involving the overexpression or suppression of the sweetpotato *IbNOI* gene in transgenic plants will provide valuable information for the development of crops with enhanced tolerance to a variety of stresses.

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초록 : 고구마에서 질소 유도성 유전자의 분리 및 특성분석

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본 연구에서는 산화질소 유도성(nitric oxide-induced, NOI) 유전자를 건조 처리된 고구마 실뿌리의 EST 라이브러리에서 분리하였다. 분리된 IbNOI 유전자의 cDNA는 712 bp의 길이이며, 77개의 아미노산으로 구성되어 있었다. Blast 데이터베이스를 분석한 결과, 식물에서 보고된 NOI 단백질에 속하는 것으로 확인되었다. RT-PCR과 realtime-PCR을 통해 IbNOI 유전자의 발현수준을 고구마 식물체의 조직별로 조사한 결과, 저장뿌리와 현탁배양세포에서 높은 발현 수준을 보였다. 잎에서 산화질소를 유도하는 SNP와 화합물 스트레스들을 처리시, IbNOI의 발현이 증가함을 확인할 수 있었다. 또한 고염, 건조와 같은 비생물학적 스트레스 및 병원균 감염에 의해서도 IbNOI의 발현이 유도됨을 확인할 수 있었다. 본 연구의 결과들을 통해 IbNOI 유전자는 다양한 비생물학적 스트레스 및 병원균 감염 동안 산화질소와 연관된 조절기작을 통해 식물의 방어기능에 관여할 것으로 생각되는 바이다.