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Short Communication

Isolation and Identification of Short Term Drought-Induced Genes in *Zea mays* L. Leaves

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ABSTRACT

Drought is one of the detrimental factors that impair plant growth and productivity. In this study, we applied annealing control primer (ACP)-based reverse transcriptase PCR (polymerase chain reaction) technique to identify differentially expressed genes (DEGs) in maize leaves in response to drought stress. Two-week-old maize seedlings were exposed to drought (DT) by suspending water supply. DEGs were screened after 3 days of DT-treated samples using the ACP-based technique. Several DEGs encoding 16.9 protein, antimicrobial protein, hypothetical protein NCLIV_068840, thioredoxin M-type were identified in maize leaves under drought stress. These genes have putative functions in plant defense response, growth and development. These identified genes would be useful for predictive markers of plant defense, and growth responses under drought stress in plants.

(**Key words** : Abiotic Stress, Drought, Gene, Maize)

Ⅰ. INTRODUCTION

Plant growth and productivity are adversely affected by abiotic stresses, such as drought, salt, extreme temperature (Mittler, 2006). Among the abiotic stresses, drought is one of the most critical limiting factors that reduce crop yield in agricultural regions. Due to effect of insufficient rainfall and/or deficient soil moisture the water resources for agricultural uses are limiting gradually (Zhou et al., 2017). Therefore, identification of drought responsive candidate gene and/or mechanisms development of drought-tolerant cultivars is intense research interest. Maize (*Zea mays* L.) is one of the important crops globally, considered as an excellent sources of food crop, livestock feed and biofuels. However, this crop severely suffered by drought stress, causing to annual yield reduction that approximately with 22 million tones globally (Shou et al., 2004). In addition, the frequent water shortages are mostly found at seedling stages of maize that can confer negative effect on next developmental stages. Drought stress inhibits ear and silk growth resulting delay in silk emergence and kernel development which leads to finally reduce grain yield in maize (Li et al., 2007).

Plants exposed to drought induce a series of physiological, biochemical and molecular alterations. Advanced genomic technologies provide to help the understanding of global gene expression in plants. Extensive studies of genes expression helps to identify several target genes in the regulation of cellular processes including signal transduction, and stress tolerance which have previously been found to be induced in several plants including *Arabidopsis* (Swindell, 2006), maize (Kakumanu et al., 2012), alfalfa (Rahman et al., 2015; 2016), and barley (Wehner et al., 2016). Several molecular techniques are used to analyze gene expression in plants. For instance, microarray and the Affymetrix GeneChip were applied to explore the effect of abiotic stimuli on gene expression (Zinselmeier et al., 2002), and cDNA microarray was applied to study transcriptional profiles in maize karnels under drought stress (Yu and Setter, 2003). Serial analysis of gene expression (SAGE) is a very efficient method that applied to analyze gene expression profile in *Arabidopsis* and rice plants (Sharma et al., 2007).

In addition, to screen differentially expressed genes (DEGs), the polymerase chain reaction (PCR) based technique has been used, but it has been found to provide significant false positive

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rates and poor reproducibility (Liang and Pardee, 1995). However, an annealing control primer (ACP) based system can provide the solution, which increase annealing specificity to the template and amplifies only the genuine gene products. This technique would be useful to identify DEGs induced by multiple abiotic stresses in plants. The objectives of this study are: i) to isolate the drought induced genes in maize leaves, and ii) to identify of key DEGs using an annealing control primer (ACP) based approach in maize crop exposed to drought.

Ⅱ. MATERIAL AND METHODS

1. Plant materials and drought treatment

Maize (*Zea mays* L. cv. Gwangan) seeds were obtained from Grassland and Forages Division, National Institute of Animal Science (NIAS), Rural Development Administration (RDA), Cheonan, Korea. Maize seedlings were grown in plastic pots containing Agriculture Nursery Medium (Biomedia, Korea). Culture temperature was maintained at 25 °C with of 400 μmol m^{-2} s^{-1} light intensity and 14-h/8 h light and dark cycle. Seedlings were maintained about 80% field capacity up to 2 weeks (V2 stage), followed by drought treatment was initiated by water suspending while control plants were irrigated as regular basis. After 3 days of treatment the seedlings were suffered drought. Leaves were excised and collected that had been stressed for three days, immediately frozen in liquid nitrogen and kept at -80 °C until use.

2. Extraction of total RNA from maize leaves and first strand cDNA synthesis

Total RNA was isolated from maize leaf tissue of treated and control plants using plant RNeasy mini kt (Qiagen USA). The RNA sample was used for the first stand cDNA synthesis by reverse transcriptase. The reserve transcription reaction was performed for 1.5 h at 42°C using 20 μl containing 3 μg of the purified total RNA, 4 μl of 5x reaction buffer (Promega, USA), 5 μl of dNTPs (2 mmol each); 2 μl of 10 μM dT-ACP1 [5′-CTGTGAATGCTGCGACTACGA TIIIIIT(18)-3′]; 0.5 μl of RNasin RNase Inhibitor (40 U/μl; Promega); and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl; Promega). Following first-strand cDNAs synthesis, all samples were diluted individually by adding 80 μl of ultra-purified water subsequently prepared for GeneFishingTM technique.

3. ACP-based GeneFishingTM reverse transcription

Gene fishing DEGs-kit (Seegene, Korea) was applied for gene isolation, followed by the amplifications was performed by annealing control primer (ACP)‐based PCR technique). Briefly, the second strand cDNA synthesis was conducted using PCR protocol and experimental method of Lee et al. (2012). The amplified PCR products were collected then

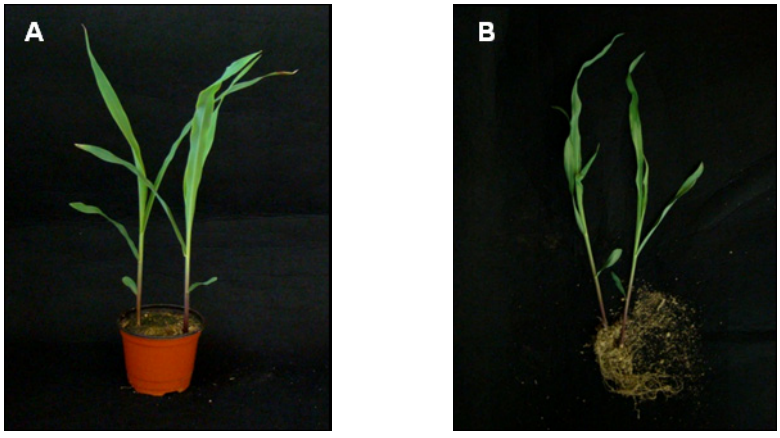

Fig.1. Morphological changes of maize under drought stress. Drought was initiated by suspending water supply for 3 days, (A) Photograph showing the control plant irrigated as regular basis, and (B) after 3 days of drought treatment.

separated using 2% agarose gel containing RedSafe™ Nucleic Aacid Staining Solution (5 μl/100 ml; ABC Scientific, USA).

4. Cloning of gene and sequencing

Drought-induced DEGs were extracted from the gel by using the GENCLEAN II Kit (Q-BIO gene, USA), followed by cloned into a TOPO TA cloning vector (Invitrogen, USA) according to the manufacturer's protocol. The sequences of cloned plasmids were carried out by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA) using the M13 forward primer (5'-CGCCAGGGTTTTCCCAGTCACGA-3') and M13 reverse primer (5'-AGCGGATAACAATTTCACACAGGA-3'). Finally, the sequences of specific genes were confirmed in NCBI database using Blast tool

Ⅲ. RESULTS AND DISCUSSION

In order to identify the drought-responsive DEGs, an annealing control primer (ACP) based approach was used. Maize plant was treated by drought stress. Morphological changes were observed between treated and control plants (Fig.1), and temporal expression of genes was analyzed in drought-treated maize plants. Using GeneFishing primers (GFPs), we obtained 7 GFPs showed differentially expressed DNA bands, and 5 GFPs encoding hypothetical 16.9 k protein (DEG2), antimicrobial protein (DEG5), hypothetical protein NCLIV_068840 (DEGs; 9, and10) and thioredoxin M-type (DEG 13) were identified (Fig. 2; Table1). Their overall functions are closely interrelated in their potential role in stress tolerance. In the following sections, we discussed these genes

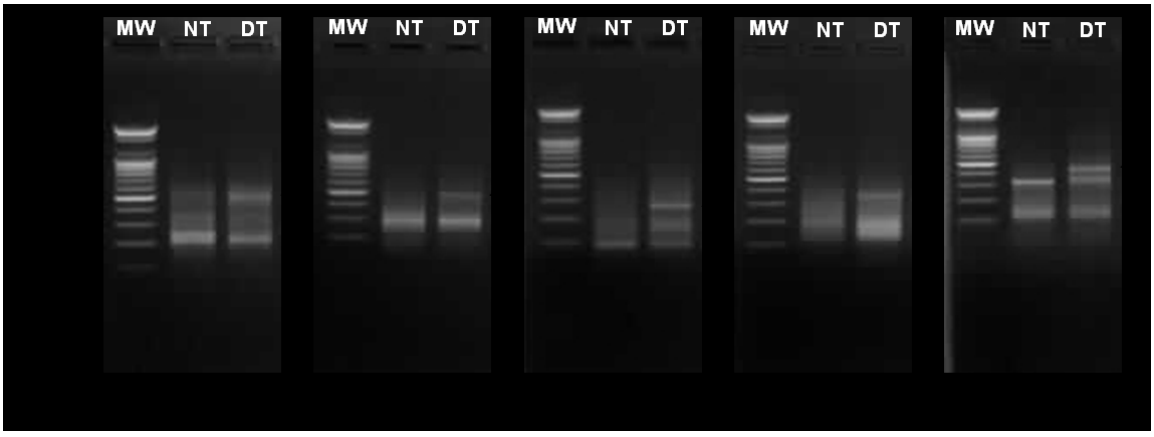

Fig.2. Annealing control primer (ACP) based approach. Agarose gel image shows differentially expressed genes (DEGs) response to short-term drought (DT) stress. Arrows indicate the DEGs compared to non-treatment. MW, molecular weight size marker; NT, non-treatment; DT, drought treatment (3 days); GFP, gene fishing primer

Table 1. Drought (Dt)-stress induced differentially expressed genes (DEGs) in maize leaves identified by sequence analysis. The sequences were searched by BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/).

DEG No	GFPs	Identity BLAST (blastx)	Total score	E value	Identity	Accession
DEG 2	U2	Hypothetical 16.9 k protein [*Salmonella typhimurium* plasmid NTP16]	251	6.00E-82	91%	JQ1541
DEG 5	U7	Antimicrobial protein [Uncultured bacterium]	302	3.00E-98	88%	AQW80360.1
DEG 9	U13	Hypothetical protein NCLIV_068840 [*Neospora caninum* Liverpool]	229	2.00E-73	100%	CCA30004.1
DEG 10	U15	Hypothetical protein NCLIV_068840 [*Neospora caninum* Liverpool]	244	3.00E-79	100%	CCA30004.1
DEG 13	U18	Thioredoxin M-type [*Zea mays*]	80.1	9.00E-15	100%	ACG24386.1

in molecular and physiological studies revealed in plant system.

We identified 16.9 k protein (DEG2) induced by drought stress (Fig.2). It has been previously found to be induced the 16.9 k protein (DEG2) that involved in phenazine biosynthesis in bacteria (León-Martínez et al., 2012). Phenazines comprised of a large group of nitrogen-containing heterocyclic compounds associated with the response of eukaryotic host and host tissues. In our study, up-regulation of 16.9 k protein during drought stress indicates its (DEG2) responsiveness to abiotic stress. It has been studied that the phenazine influenced growth and elicited systemic resistance in plant (Pierson and Pierson, 2010). Plants are excellent source of antimicrobial materials; an antimicrobial protein (DEG5) identified in response to drought stress in maize leaves. This is not surprising because antimicrobial genes/enzymes were found to be induced by abiotic stresses in plants. For instance, a novel small antimicrobial protein *LJAMP1* expressed in Motherwort (*Leonurus japonicus*) that conferred disease resistance in tobacco plant (Yang et al., 2007). Another antimicrobial protein gene, *CaAMP1* (*Capsicum annuum ANTIMICROBIAL PROTEIN1*) significantly induced by abiotic elicitors as well as pathogen response that conferred broad-spectrum of resistance to bacterial pathogen (Lee et al., 2008).

We identified two homologs as hypothetical protein NCLIV_068840 (DEGs 9, and 10) which were induced by drought stress in maize leaves. A similar observation has been found in creeping bentgrass whereas hypothetical protein NCLIV_068840 was up-regulated by drought stress that involved in drought tolerance mechanisms (Merewitz, 2012).We also identified thioredoxin M-type (Trx-m; DEG 13), a major regulator of Calvin cycle enzyme. *Trx-m* is an essential redox regulator in the light regulation of photosynthetic mechanism (Wang et al., 2013). In this study, up-regulation of *Trx-m* gene in response to drought stress suggests that this gene may regulate the activity of other chloroplastic genes in a light-dependent approach. In addition, *Trx-m* gene can impact on plant growth and development. Recently, *Trx-m*-deficient mutants of *Arabidopsis* were used to evaluate the physiological role of *Trx-m* gene; the deficiency of *Trx m* gene greatly impaired plant growth and reduced the rate of CO_2 assimilation (Okegawa and Motohashi, 2015).

Ⅳ. CONCLUSION

In this study, several genes encoding 16.9 k protein, antimicrobial protein, hypothetical protein NCLIV_068840, thioredoxin M-type were identified using ACP-based RT-PCR approach induced by drought stress in maize leaves. These genes were significantly induced by drought stress in maize leaves. These genes were up-regulated which were associated with defense response, plant growth and development. These up-regulated expressions might be used as predictive markers in plant defense and abiotic stress response.

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