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The Function of *ArgE* Gene in Transgenic Rice Plants

Jia Guo, Eun Soo Seong, Joon-Hyeong Cho[1] and Myeong-Hyeon Wang*
School of Biotechnology, Kangwon National University, Chuncheon, Kangwon 200-701, Korea
[1]Department of Plant Biotechnology, Dongguk University, Seoul 100-715, Korea

Abstract - We carried out to study the function of *ArgE* in transgenic rice plants, which were confirmed by PCR analysis and hygromycin selection. Transgenic rice plants were with selectable marker gene (HPT) inserted in genome of the rice. Southern analysis with *hpt* probe confirmed by two restriction enzymes that copy numbers of the selectable gene was introduced into the plant genome. We displayed that the relationship between drought stress and *ArgE* gene with the overexpressing rice plants. From this result, we observed that the degree of leaves damage has no difference in control and transgenic lines. The total RNAs were extracted from 6 weeks-seedling in normal condition in order to examine their expression levels with *ArgE*-overexpressed transgenic rice. In particular, expression patterns of genes encoding enzymes involved in abiotic stress, including drought and salt stresses. *OsGF14a* and *OsSalt* were investigated by reverse transcription-PCR (RT-PCR). Expression levels of the *OsSalt* gene decreased significantly in transgenic rice plants compared to control plant. However, ion leakage measurement did not demonstrate any leaves damage change between control and *ArgE* transgenic plants exposure to mannitol treatment. These results suggest that expression of the *ArgE* is not involved in tolerance for drought stress in rice but may play a role of signaling networks for salt-induced genes.

Key words - *ArgE*, Hpt gene, Ossalt, OsGF14a, RT-PCR

Introduction

Rice has become a model monocot system for genetic and functional genomic studies and is the important crop for more than half of the world population. More efforts have been proceeded towards the improvement of agronomic traits of rice by biotechnological techniques for recent years. Asian cultivated rice (*Oryza sativa* L.) composed of two major groups such as the subspecies names indica (*Oryza sativa* ssp. *indica*) and japonica (*Oryza sativa* ssp. *japonica*). Many subspecies-specific genes were known to the sequence divergence revealed by whole genome sequencing of the two subspecies of cultivated rice (Zhang *et al.*, 2005).

Many attempts have been produced to introduce many genes into plants. Plant gene transformation was proceeded by particle bombardment with multiple gene constructs (Romano *et al.*, 2003; Kim *et al.*, 2003; Wang *et al.*, 2003; Loc *et al.*, 2002). *Agrobacterium*-mediated transformation was made good with multiple plasmids (Ebinuma *et al.*, 2001; Daley *et al.*,1998; Chang *et al.*, 2002), and introducing internal ribosome entry site by which multiple gene expression can be controlled as a single transcript unit like in prokaryotes (Urwin *et al.*, 2000). However, gene silencing and co-suppression due to high copy number of genes or position effects (Romano *et al.*, 2003; Stam *et al.*, 1997) are brushed against transgenic plants transformed by particle bombardment.

Many genes that were functional involved in drought stress in wild habits are strongly expressed in crop plants. Crop plants show different responses when they are subjected to drought. However, it should be noted that some of these responses are death-directed responses. *ArgE* is N-acetylornithine deacetylase, and catalyses hydrolysis of the N-acetyl group of N-acetylornithine to give ornithine and acetate in Escherichia coli (Meinnel *et al.*, 1992). This reaction is the final step of the acetyl cycle for arginine synthesis, which serves to incorporate the carbon skeleton of glutamate into the urea cycle (Kawasaki *et al.*, 2000). The whole sequence of the cDNA has no extra sequence for translocation of the translated peptide into an organelle (Kawasaki *et al.*, 2000). One of these drought-induced peptides (DRIP), DRIP-1, is produced in abundance. The DRIP-1 cDNA has been cloned based on its N-terminal sequence (Kawasaki *et al.*, 2000), and is a homologue of the ArgE/DapE/Acy1/CPG2/Yscs family (Biagini and Puigserver, 2001).

*Corresponding author. E-mail : mhwang@kangwon.ac.kr

Rice is a good model to study plant gene transformation and function in monocots. Having a simple and efficient method to introduce foreign gene by *Agrobacterium* mediated transformation would be helpful in producing transgenic plants inserted gene with new function. In this study, we displayed that the relationship between drought stress and *ArgE* gene with the overexpressing rice plants. In particular, expression patterns of genes encoding enzymes involved in abiotic stress, such as OsGH14a and OsSalt were investigated by reverse transcription-PCR (RT-PCR).

Materials and Methods

Agrobacterium-mediated transformation

Single bacterial colony of *Agrobacterium* strain AGL1 was incubated in AB medium (AB buffer and salt, 5g/l glucose, 15g/l Bacto-agar, 50mg/l spectinomycin, 10mg/l tetracycline, pH 7.2) at 28 ℃ for 72 h. Bacterial cells were collected by scraping and resuspended in AAM medium (AA macro and micro salt, Amino acid stock, MS vitamins, 500mg/l casamino acid, 68.5g/l sucrose, 36g/l glucose) containing 100mM acetosyringone with the concentration of *Agrobacterium* to 5×10^9 cells/ml. The *Agrobacterium* strain AGL1 was mixed at 1:1 ratio before being used for transformation.

Embryogenic calli from the scutellum of mature seeds, "Ilmibyeo" was used for transformation according to method of Hiei *et al.* (1994) with slight modification. Embryogenic calli were mixed with *Agrobacteria* in 25ml AAM solution containing around 5×10^9 cell / ml and incubated for 20 min at room temperature. The calli were then plated on NB3 medium (N6 4g, casein 0.3g, proline 2.9g, sucrose 30g, 2.4-D 2ml, glutamine 0.5g, N-B micro 1ml, N-B vts 1ml, myo-inositol 0.1g, pH 5.8, gelrite 2.5g) for co-cultivation at 25℃ in dark condition for 3 days. After washing the *Agrobacteria* using cefotaxime solution (250mg/l in distilled water), the calli were transferred to 2N6-CH medium (2N6 medium supplemented with 250mg/l cefotaxime, 50mg/l hygromycin B) and cultured at 27 ℃ in dark condition for 3 weeks. Selection was done with hygromycin B resistance primarily during the first five weeks (callus proliferation stage) after transformation treatment. Selected calli were subcultured 2 weeks on fresh 2N6-CH medium and further grown for 3 weeks and transferred to MSRK5SS medium (MS 4.4g, NAA 1mg, Kinetine 5mg, sucrose 20g, sorbitol 30g, proline 0.5g MES 0.5g, pH 5.8, gelrite 4g, cefotaxium 250mg/l, hygromycine 50mg/l) for second selection and regeneration for 4 weeks at 27℃ under continuous light condition. The regenerated shoots were transferred to MS medium (MS salts and vitamins, 30g/l sucrose, 2g/l phytagel, pH 5.8) for root induction for 2 weeks before acclimation in a Magenta box containing 0.1% hyponex solution (Hyponex Co., Imlay City, MI 48444) for 1 week in a culture room. The plantlets were then transplanted to a Wagner pot (1/5000a) in a greenhouse for subsequent growth.

PCR analysis

The genomic DNA isolated from wild type and putative transgenic plants were used as template to amplify *hpt* gene. The primers were designed for a 650 bp of *hpt* genes. The primer sequences used were as follows: *hpt* forward, 5' -ATCCTTCGCAAGACCCTTCCT-3' · and *hpt* reverse, 5' -GGTGTCGTCCATCACAGTTTG-3' . The PCR cycle program consisted of initial denaturation at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 90 sec, followed by a final extension at 72℃ for 7 min. The PCR product was electrophoresed in 1% agarose gel.

Southern blot

Transgenic plants were analyzed by PCR and Southern hybridization for the integration of the transgenes. For Southern blot analysis, 10μg of purified DNA was completely digested with 20 U of *Eco* RI and *Hind* III. DNA fragments were resolved on 0.8% agarose gels and blotted on N^+ Hybond nylon membranes. Radiolabeled probe of *HPT gene* sequence was prepared with the ^{32}P.

Measurement of electrolyte leakage

Leaf damage was observed on the leaf discs from five-week-old seedlings after it was immersed in 1 M mannitol solution for at 4 days. After 2 days, ion leakage was measured using an electrical conductivity meter (model 1: 455C, Istek, Seoul, Korea). Samples were collected in autoclaved 15ml falcon tubes and the total content of electrolytes in each sample was determined. The tests for visible damage analysis by mannitol applications were repeated in triplicate (Li *et al.*, 2006).

RT-PCR

Total RNA was isolated from the leaf discs of transgenic rice using TRI-reagent® according to the manufacturer' s instructions (MRC, USA). Total RNA was treated with 1 U DNase for 10 min at 37℃ and subjected to a second round of TRI-reagenet

Table 1. Primers used for reverse transcription-PCR

Gene	Forward primer (5' -3')	Reverse primer (5' -3')
OsActin	ATGGCTGACGCCGAGGAT	TTAGAAGCATTTCCTGTG
ArgE	ATGAAAAACAAATTACCGCC ATT	TTAATGCCAGCAAAAATGGTA
OsGF14a	TGAGCGTTACGAGGAAATGG	GATGGGACAAGGTGGGAATC
OsSalt	ACATCGGTGTGGATGGACAG	CAATTGCGTCGATAAGCGTT

purification. From the DNase-treated total RNA (1μg), first-strand cDNA was synthesized using the AccuPower® PCR PreMix (Bioneer, Korea) containing oligo (dT) primers, and Moloney murine leukemia virus reverse transcriptase (Invitrogen, USA). The primers used for reverse transcription-PCR were shown in Table 1. The PCR reaction was carried out as follows: initial 5 min denaturation at 94℃; followed by 28 cycles of 94℃, 30s; 60℃, 30s; and 72℃, 30s; and a final 7 min at 72℃. Twelve μl samples of the reaction products were separated on 1% agarose gels and visualized after staining with ethidium bromide. All experiments were preformed in triplicate.

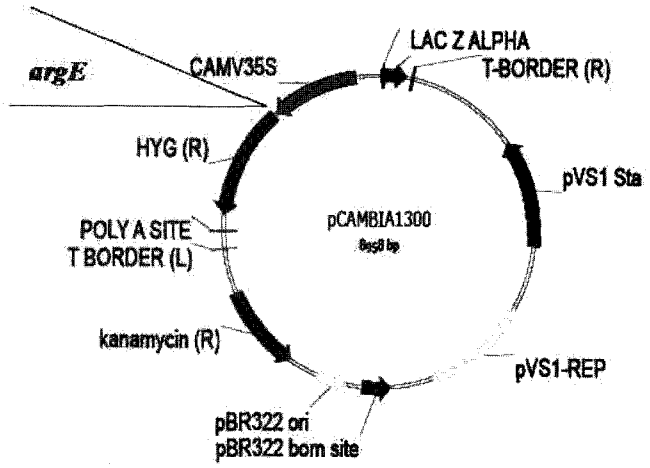

Fig. 1. Vector construction of the T-DNA region of pCambia1300 plasmid with *ArgE* gene.

Results and Discussion

Agrobacterium-mediated transformation of rice

An *ArgE* gene was cloned into pCambia1300 harboring hygromycin resistance gene (HPT) (Fig. 1). In the PCR analysis of transformation with primers *HPT* (Forward & Reverse), bands were observed both in plantlets regenerated on a medium with 50mg/l of hygromycin only, and in control plantlets. The fragments, 650 bp in size, were observed in the plantlets transformed and regenerated from callus (Fig. 2). We also examined that selectable marker gene (HPT) inserted in genome of transgenic plants. Southern analysis with *hpt* probe confirmed by two restriction enzymes that copy

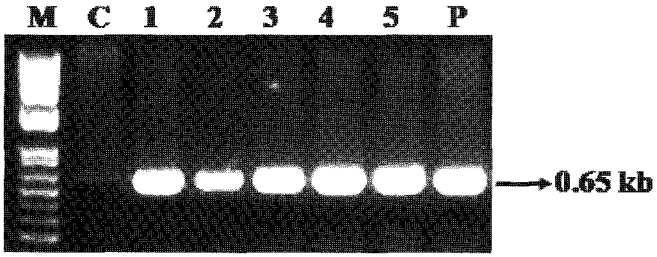

Fig. 2. Detection of the 650 bp *hpt* gene amplified by PCR using DNA isolated from *ArgE* transgenic rice plants. PCR products of *hpt* gene from T0 plants obtained by first selection scheme (Hyg50). M: 1 kb plus DNA ladder marker, C: non-transgenic rice plant, 1-5: transgenic rice, P: positive control by PCR from isolated plasmid DNA.

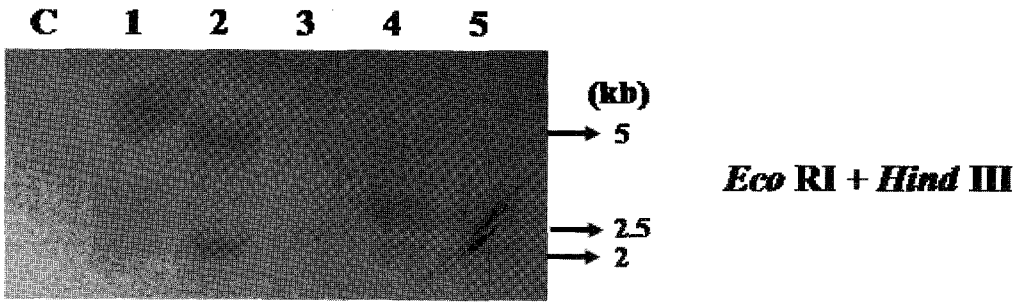

Fig. 3. Southern blot of rice transgenic plants transformed with *HPT* genes using second selection scheme. Genomic DNAs from the leaf of five (1-5) transgenic lines obtained by second selection scheme and digested with *Eco* RI and *Hind* III. C, Genomic DNAs from untransformed control plant; 1-5, Genomic DNAs from transgenic rice plants.

numbers of the selectable gene was introduced into the plant genome (Fig. 3). Some of the transformed plantlet lines detected multiple bands in southern blot. The assay used for the hygromycin phosphotransferase (HPT) enzyme indicates the effective nature of the selection system. Rice transformation has also been successfully accomplished with immature embryos or embryogenic calli, but it is only recently that biolistic transformation of rice has been shown to be effective (Zhang *et al.*, 1997). More recently, other methods such as *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994) and electroporation of intact seed embryo cells (Xu and Li, 1994) have been published.

Drought stress of transgenic rice plants

Plant tissue damage is caused mainly in conditions of stress. Cell membrane stability can be estimated through electrolyte leakage following exposure to physical stress factors (Ferreira *et al.*, 2006). Ion leakage increased about 2- and 3- fold on average relative to undisturbed control plants. In our study, the effect of 1 M mannitol treatment on leaf discs has been used to test tolerance to drought stress (Fig. 4). Control plant and *ArgE* transgenic plant showed some differences when treated with distilled water. However, ion leakage measurement did not demonstrate any leaves damage change between control and *ArgE* transgenic plants exposure to mannitol treatment. This result suggests that expression of the *ArgE* is not involved in tolerance for drought stress in rice. Since the water potential equals to the sum of the osmotic and turgor pressures, the decrease of the leaf water content causes a decrease of the turgor pressure and consequently the water potential (Kramer and Boyer, 1995). The primary structure of the polypeptide deduced from the DRIP-1 cDNA exhibited homology to those of the proteins belongs to the ArgE/DapE/Acy1/CPG2/YscS family (Boyen *et al.*, 1992; Vongerichen *et al.*, 1994). DRIP-1 had two consensus motifs for this family (Two-arrowhead lines a and b); the former contained a histidine residue for metal ion-binding. The enzymes belonging to this family are acetylornithine deacetylase (*ArgE*) (Meinnel and Yokota,1992), amino acylase-1 (Acy 1) (Mitta *et al.*, 1992), carboxy peptidase G2 (Cpg2) (Minton *et al.*, 1986) and carboxy peptidase S (YscS) (Spormann *et al.*, 1991). Since N-acetylornithine-glutamate acetyltransferase is found in the chloroplasts of pea leaves (Taylor and Stewat, 1981), Drip-1 in the cytosol may be involved in additional sequestration of the glutamate skeleton into the urea cycle. In addition to the induction of DRIP-1 during the drought stress in wild watermelon, dramatic changes in the amino acid content and in its composition were observed (Kawasaki *et al.*, 2000).

Gene expression in transgenic plants

To evaluate the genes related to abiotic stress sought from NCBI homepage, two genes (accession no. P24120 & Q06967) were selected for RT-PCR. In order to examine their expression levels with *ArgE*-overexpressed transgenic rice, total RNAs were extracted from 5 weeks-seedling in normal condition. The results showed that transcriptional level of OsGF14a (Q06967), a drought

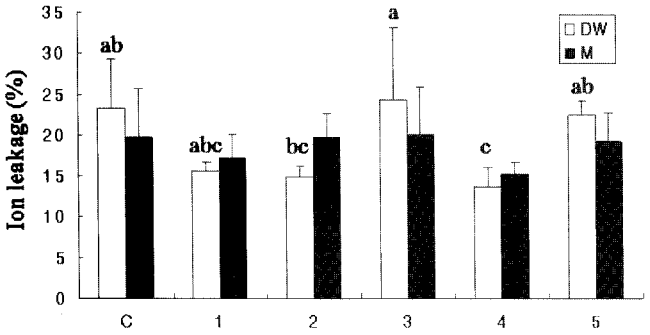

Fig. 4. Ion leakage of control and transgenic rice plants treated with drought stress. Control plants and transgenic rice lines 1, 2, 3, 4, and 5 were compared for drought-stress tolerance. The leaf disc were immersed in 1 M mannitol solution for 4 days and then exposed to 24℃ for 24 h. Ion leakage was calculated from the percentage by damaging plant tissues 4 days after the drought treatment. Values not sharing a common superscript letter are different (n = 3; $P < 0.05$).

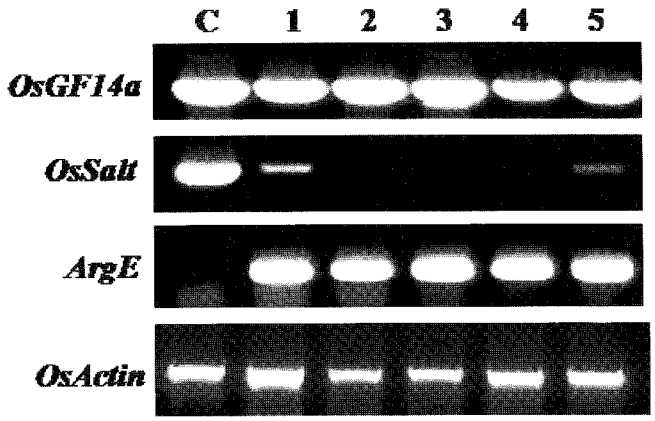

Fig. 5. Expression levels of abiotic stress-related genes in *ArgE*-overexpressed plants by RT-PCR. Total RNAs were extracted from leaf tissues of the control and the transgenic plants. The *OsActin*, *ArgE*, *OsSalt* and *OsGF14a* were measured the expression level using RT-PCR. C; non-transformed plant, 1-5: transgenic rice plants.

stress response gene, was not changed (Fig. 5). The *OsSalt* gene (P24120) expression levels changed significantly, which was reduced in transgenic rice plant compared to control plant. These results suggest that expression of the *ArgE* is not involved in tolerance for drought stress in rice but may play a role of signaling networks for salt-induced genes. However, we still do not know how transcriptional expression of the *OsSalt* gene was down-regulated in the *ArgE*-overexpressed rice plants. In drought, electrons that have no productive destination in the electron transport chain are directed to the reduction of molecular oxygen to form superoxide and hydrogen peroxide (Miyake and Yokota, 2000). The remaining 50% is the flux in cyclic electron transfer in PSII (Miyake and Yokota, 2001). A salT was selected and found to contain an open reading frame coding for a protein of 145 amino acid residues. Generally, no induction was seen in the condition of the stress should affect all parts of the plant. The organ-specific response of salT was corresponded to the current of Na^+ accumulation during salt stress (Claes *et al.,* 1990). A rice cDNA which is similar to the bovine brain-specific 14-3-3 protein (an activator protein of tyrosine) gene was cloned. This gene indicated a similarity to a potent inhibitor of Ca^{2+}-phospholipid-dependent protein kinase C. Expression of this gene is regulated by abiotic stresses (Kidou *et al.,* 1993).

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