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Isolation of salt-inducible genes related to signal transduction in barley by differential display

A Ueda[1], WM Shi[2] and T Takabe[2]

[1]*Bioscience Center, Nagoya University, Chikusa, Nagoya 464-8601, Japan.*

[2]*Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan. h44854a@nucc.cc.nagoya-u.ac.jp*

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Introduction

To survive under various environmental stresses, plants developed signal transduction systems to acquire the tolerance. It is important how quickly and efficiently plants sense outer stimuli. Generally, it is considered that a receptor or sensor in cell membranes catch/ percept the signal from outside and then phospholyrate its target protein inside cell. It was reported that a histidine kinase plays a role of osmosensor and its expression was induced at 10 minutes by salt stress (Urao et al., 1999). The transcription levels of mitogen activated protein kinase (MAP kinase) and phosphatidylinositol-4-phosphate-5-kinase (PIP-5 kinase), one of the components in signal transduction cascade, were also increased by salt stress (Mikami et al., 1998; Munnik et al., 1999). However these components were a part of complicated system, the information involved in signal transductions under salt stress is still limited.

Since expression of mRNAs of components involved in signal transduction is low, their detection is sometimes difficult. Differential display is one of the mRNA finger print techniques and enables to detect the genes expressed in lower levels because of the use of RT-PCR. In this work we isolated the candidates of salt stress-responsive genes related to signal transduction in barley by differential display.

Materials and Methods

1. Plant materials

Barley (*Hordeum vulgare* L. cv. Haruna-nijyo) was hydroponically grown with Hoagland nutrient solution in a growth chamber (13 h light period, 100μmol m^{-2} s^{-1}, 25 °C, humidity 70%; 11 h dark period, 22 °C, humidity 75%). Three-week-old plants were treated with 100 mM and then 200 mM NaCl for 3 days each, and then roots were harvested and stored at –80 °C until RNA extraction for differential display.

2. Differential display

First strand cDNA was synthesized from 5μg poly$(A)^+$ RNA using reverse transcriptase (Superscript II; GIBCO BRL) and random hexamer (pd$(N)_6$; Amersham Pharmacia Biotech). The PCR reaction mixture contained 1 ng cDNA, 1μM randomly amplified polymorphic DNA (RAPD) (12 mer, Common's primer; Bex), 0.2 mM dNTP, 1 unit Amplitaq DNA polymerase (Perkin-Elmer) and PCR buffer in a final volume 20μl. After denaturing at 94 °C for 5 min, the PCR reaction was carried out for 40 cycles of 94 °C for 1 min, 35 °C for 1 min

and 72 °C for 2 min with an additional extension at 72 °C for 5 min. PCR was performed at least 3 times per each primers.

3. DNA sequencing and analysis

The DNA sequencing was determined using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and a DNA sequencer (ABI PRISM 373A). All sequences were compared to databases by the BLASTx algorithms. A deduced amino acid sequence homology between a known sequence and an EST was considered as significant when the BLASTx BLOSUM 62 score was more than 100.

4. Northern hybridization

Fifteen μg total RNA was separated on 1.2% agarose gel containing 0.66 M formaldehyde and blotted onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech). The PCR fragments obtained by differential display were used as a probe. Hybridization was performed in a solution containing 6X SSC, 5X Denhardt's solution, 0.1% SDS and 0.1 mg/ml denatured herring sperm DNA. The membrane was washed in 6X SSC twice for 30 min. All hybridizations and washings were performed at 65 °C.

Results and Discussion

We performed differential display using 480 species RAPD primer and total 218 candidates were detected as salt-inducible genes (Ueda et al., submitted). Classification of their putative functions showed that 13% of them are related to signal transduction (27 candidates). Eight and 7

Table 1. Catalog of cDNA detected by differential display in barley.

Category	*Numbers of detected genes*	*Best homology to database*
serine/ threonine protein kinase	8	MAP kinase[2], casein kinase[1], putative protein kinase[1], etc.
receptor protein kinase	7	putative receptor protein kinase[3,4]
transcription regulator	5	RUSH-1 alpha[1], SET-domain regulator[1], putative ATPase[1], etc.
others	7	protein phosphatase 2A[2], SCARECROW[1], EPS15[5], AMSH[5], etc.

Sources in database are [1]*A. thaliana*, [2]*O. sativa*, [3]*Z. mays*, [4]*N. tabacum* and [5]*H. sapiens*.

candidates belong to serine/ threonine protein kinase (MAP kinase, casein kinase and 6 putative protein kinases) and receptor protein kinase (RPK) family, respectively (Table 1). In plants, it is considered that serine/ threonine kinase occupies more than 90 % in all kinases. MAP kinase, one of serine/ threonine kinases, was detected in this study and the induction of its mRNA is enhanced by various stresses (Jonak et al., 1996; Kovtun et al., 2000). RPK has a membrane spanning region and kinase domain, and its putative function is transduction of outer signal to inner target protein. One of them (a homologue of Arabidopsis RPK, Accession No. S71277) was strongly induced by salt (data not shown). Some transcription regulators were detected in salt stressed-barley. A putative ATPase was found to have three leucine zipper motif and similar to SNF2 in yeast.

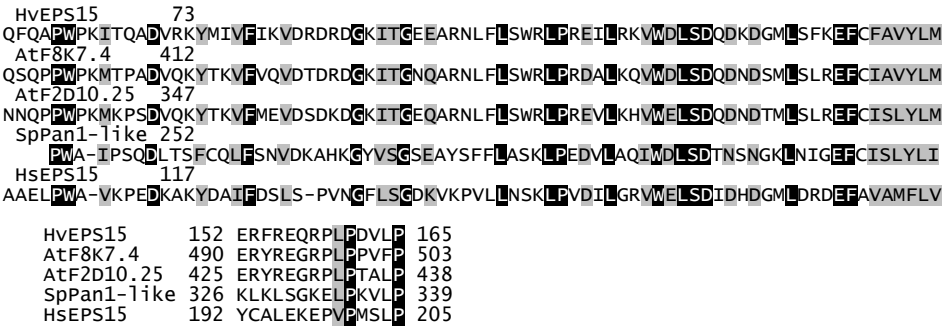

Fig. 1 Alignment of HvEPS15, Arabidopsis EPS15s, fission yeast Pan1-like protein and human EPS15. Identical and similar amino acid residues were indicated with black and gray boxes, respectively.

Furthermore, we clarified that the levels of the mRNAs of PIP-5 kinase, SCARECROW, EPS15 (HvEPS15) and AMSH were increased by salt stress. Barley EPS15 has homology to human EPS15, fission yeast PAN1-like protein and putative Arabidopsis EPS15s (Fig. 1). EPS15 has an EH-domain and interacts with epidermal growth factor receptor (EGFR) in animal cells (Fazioli et al., 1993). The interactions between EPS15 and EGFR mediated by tyrosine phospholyration are implicated in endocytosis and synaptic vesicle recycling. It was also reported that EPS15 is required for ligand-regulated endocytosis (Confalonieri et al., 2000). The transcription level of *HvEPS15* mRNA was induced by salt and cold stress (Fig. 2)

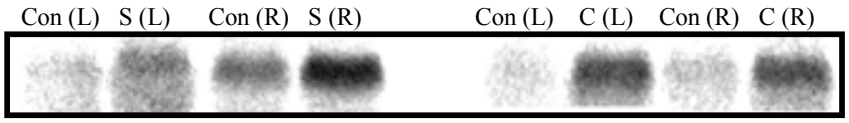

Fig. 2 Northern blot analysis of barley EPS15 homologue. Plants were treated with 200 mM NaCl for 48 h or transferred at 4 °C for 48 h. Con, control; S, salt stress; C, cold stress; L, leaves, R, roots.

Moreover, AMSH (containing SH3 domain) and dynamin (containing GTPase domain) were also detected by differential display and they were considered to be components invovled in endocytosis. These results indicated that the active of endocytosis and synaptic vesicle recycling occur under salt stress.

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