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Isolation of novel early salt-responding genes from wheat (*Triticum aestivum* L.) by differential display

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Abstract We isolated five cDNAs for salt-stress responding genes from common wheat, *Triticum aestivum* L., seedlings treated with 0.15 M NaCl for 2 h by using the modified differential display method. The clones were collectively named WESR (Wheat Early Salt-stress Responding) genes. WESR1 to 3 were isolated from leaves, and WESR4 and 5, from roots. Induction of the gene expression by NaCl treatment for 2 h was confirmed by Northern blot analysis. WESR gene transcripts accumulated from 1.3 to 2.6-fold over the controls. Based on nucleotide and deduced amino acid sequence analysis, WESR4 showed homology to a cDNA clone isolated from barley containing the zinc-finger motif. The deduced amino acid sequence of WESR5 indicated homology to the glucose-6-phosphate dehydrogenase (G6PDH) reported in potato and alfalfa. Other WESR clones did not show homology to any genes of known function, and all WESR clones are novel salt-stress responding genes.

Key words Salt stress · Wheat · Differential display · Zn-finger motif · G6PDH

Introduction

Salinity affects plant growth by causing high ionic concentration in the cytoplasm or low soil water potential. Excessive ions may also cause a deficiency of other ions, a phenomenon called "antagonism", as well as toxicity in the plants by damaging membranes and organelles (Epstein 1983). When plant cells are exposed to salinity, high levels of apoplastic salt concentrations alter aqueous and ionic thermodynamic equilibria, resulting in hyper osmotic stress (Skriver and Mundy 1990; Chen et al. 1994; Holappa and Walker-Simmons 1995; Werner and Finkelstein 1995), ionic imbalance, and toxicity. Ions traverse into the cells across the plasma membrane and tonoplast via transport proteins (Narasimhan et al. 1991; Binzel 1995; Binzel and Dunlap 1995; Ballesteros et al. 1996). Plants synthesize and accumulate organic osmolytes called compatible solutes, which control osmotic balance. Under the salt and water stress condition, the endogenous level of the plant hormone abscisic acid (ABA) increases (Gómez J et al. 1988). In order to understand plant responses to salt stress, it is important to identify genes involved in the salt response and to assess their individual contributions to physiological responses. In the present study, we isolated salt-stress related genes from wheat within 2 h after exposure to NaCl solution in order to investigate the early events of the response at the level of gene expression.

Materials and methods

Plant growth conditions and NaCl treatment

Seeds of *Triticum aestivum* L. cv 'Chinese Spring' were surface-sterilized with NaClO solution (effective chloride concentration 2.0%), and then sown in sterilized vermiculite. The plants were grown in a growth chamber at 20°C and 75% relative humidity with a 15-h photo period supplied by fluorescent lights (170–240 m molm^{-2s-1}). After 7 days, the seedlings were transferred to sterilized 1/5 strength Linsmaier-Skoog liquid medium (Linsmaier and Skoog 1965) adjusted to pH 5.7. The medium was aerated and exchanged every 3 days for fresh medium. Two-week-old seedlings were transferred to fresh medium supplemented with 0.15 M NaCl. After exposure to the NaCl solution for 2 h, leaves (the

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upper part of the tiller) and roots (the lower part) were collected, weighed, frozen with liquid nitrogen and stored at −80°C until further processed.

RNA extraction

The frozen leaves or roots were ground to a fine powder with a mortar and pestle in liquid nitrogen. The powder was homogenized with 40 ml of a solution of a 1:1 mixture of RNA extraction buffer [0.1 M Tris-HCl (pH 9.0), 0.3 M NaCl, 10 mM EDTA, 0.1% sodium lauroyl salcosinate, 10 mM DTT] and water-saturated phenol. The aqueous phase was separated by centrifugation (12 000 rpm, 20 min, at room temperature) and extracted with 1 vol of phenol/chloroform twice, followed by chloroform extraction. Nucleic acids were precipitated with 0.3 M sodium acetate (pH 5.2) and 0.6 vol of 2-propanol, collected by centrifugation (15 000 rpm, 20 min, at 4°C), and dissolved in distilled water. High molecular weight RNA was selectively precipitated by adding LiCl to a final concentration of 2.5 M, and recovered by centrifugation (15 000 rpm, 20 min, at 4°C). The precipitate was rinsed with 2 M LiCl and dissolved in distilled water. Poly(A)$^+$ RNA was isolated by Oligotex-dT_{30} (Takara Shuzo Co., Japan) according to the manufacturer's instructions.

In vitro translation and two-dimensional gel electrophoresis

Poly(A)$^+$ RNA was translated in an RNA-dependent wheat germ cell-free system (Watanabe and Price 1982). 0.8 μg amount of poly(A)$^+$ RNA was translated in a 10 μl reaction volume containing wheat germ extract, amino acids less Met, and 370 kBq of [^{35}S]Met. The reaction was carried out at 30°C for 30 min. Translation mixtures were subsequently digested with ribonuclease A for 15 min at 37°C. The translation products were placed on ice and immediately processed for isoelectric focusing (IEF). Two-dimensional gel electrophoresis was conducted essentially according to the procedure of O'Farrell (1975). The first-dimension IEF gel contained 1.6% (w/v) and 0.4% Ampholine pH 5–8 and pH 3.5–10, respectively, 2% Triton X-100, 8 M urea, 4% (w/v) acrylamide, and 0.01% (w/v) bis-acrylamide. The translation product was mixed with 8.3 M urea, 1% Triton X-100, and 2% Ampholine pH 3.5–10, 5% (v/v) 2-mercaptoethanol, and separated with 1 000 000 cpm per gel. The IEF gel was focussed at room temperature at 400 V for 12 h and then at 800 V for 1 h. After IEF, the gel was equilibrated by gentle shaking for 20 min in equilibration buffer containing 62.5 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, BPB (bromophenol blue), and 5% 2-mercaptoethanol before being loaded for the second dimension. The second-dimension gel, containing 13% acrylamide, was run at room temperature at 30 mA until the BPB tracking dye reached the end of the gel. The gel was stained with Coomassie Brilliant Blue, and dried with a gel drier. The dried gel was exposed to X-ray film at 4°C for 7 days.

RT-PCR and differential display

We employed the differential display method (Liang and Pardee 1992) to isolate salt-responding genes. The simplified differential display (Yoshida et al. 1994) was further modified as follows. First strand cDNA was synthesized from poly(A)$^+$ RNA (0.5 μg) by use of a $(dT)_{20}$ primer and SUPERSCRIPT™ II RNase H$^-$ RTase (Gibco-BRL). First strand cDNA was used as a template for PCR with an arbitrary 12-base primer (BEX, Japan) and *Taq* polymerase (Takara Shuzo). The PCR was performed for 35 cycles with the following temperature profile: denaturation for 1 min at 94°C, primer annealing for 1 min at 5°C below the melting temperature of each arbitrary primer, and extension for 2 min at 72°C. PCR products were separated through a 10% (w/v) polyacrylamide gel with Tris-glycine electrode buffer (Davis 1964). The gel was stained by the use of Silver Staining Kit DNA (Pharmacia) according to the manufacturer's instructions. Differential patterns of cDNA fragments between the lanes of NaCl treatment and the control were examined in the stained, dried gels. Fragments that were consistently differential in repeated experiments were excised from the dried gel, extracted by crushing in water, and re-amplified by PCR with the respective arbitrary primers. The re-amplified PCR products were used as the probes for Northern analysis.

Northern analysis

Total and poly(A)$^+$ RNA were separated on a 1.2% agarose gel by the glyoxal method (Sambrook et al. 1989). After electrophoresis, the gel was stained with ethidium bromide in order to assess the quality and quantity of the loaded RNAs, and blotted onto a nylon membrane (Zeta-probe GT membrane, BioRad) with 10 mM NaOH. The cDNA fragments were labelled with [α-^{32}P]dCTP by random priming using a *Bca* BEST™ Labelling kit (Takara Shuzo) and used as probes. Hybridization was performed with a buffer containing 0.25 M sodium phosphate (pH 7.2) and 7% SDS at 65°C. After hybridization, the membranes were washed with 20 mM sodium phosphate (pH 7.2) containing 5% or 1% SDS to control stringency. Quantitative data were obtained from the hybridization signal by the use of BAS-2000 (FUJIX) and BAStation software to sum total pixel values in equal-size areas encompassing signals.

Cloning and sequencing of the cDNAs

The cDNA fragments that showed different accumulation between the control and salt treatment by Northern analysis were cloned into pBluescript II SK$^-$ plasmid by use of the TA cloning system (Marchuk et al. 1990). The cDNA insert was sequenced by the dideoxy chain termination method (Sanger et al. 1977) with a DNA sequencer (Li-Cor, Lincoln, USA). Nucleotide sequences and deduced amino acid sequences were analyzed with a Genetyx-Mac computer program (Software Development, Japan). Homology searches of sequences in databases were conducted by use of the BLAST algorithm (Altschul et al. 1990; Gish and States 1993).

Results

Phenotypic appearance and gene expression of the NaCl-treated wheat plant

The 2-week-old seedlings were transferred to the medium supplemented with 0.15 M NaCl. No visible change was observed at 2 h after the treatment. The colour of the lower leaves turned from green to yellowish-green after exposure to the saline solution for 72 h. By 464 h, most leaves had turned yellow (data not shown).

To verify the gene expression in seedlings exposed to 0.15 M NaCl for 2 h, the *in vitro* translation products were analyzed by two-dimensional gel electrophoresis. Among 760 polypeptide spots identified in leaves, seven showed an increase, and two showed a decrease, in relative amount (Fig. 1 A). In roots, 840 polypeptide spots were observed, and 15 showed an increase (Fig. 1 B). Thus, although no visible response was

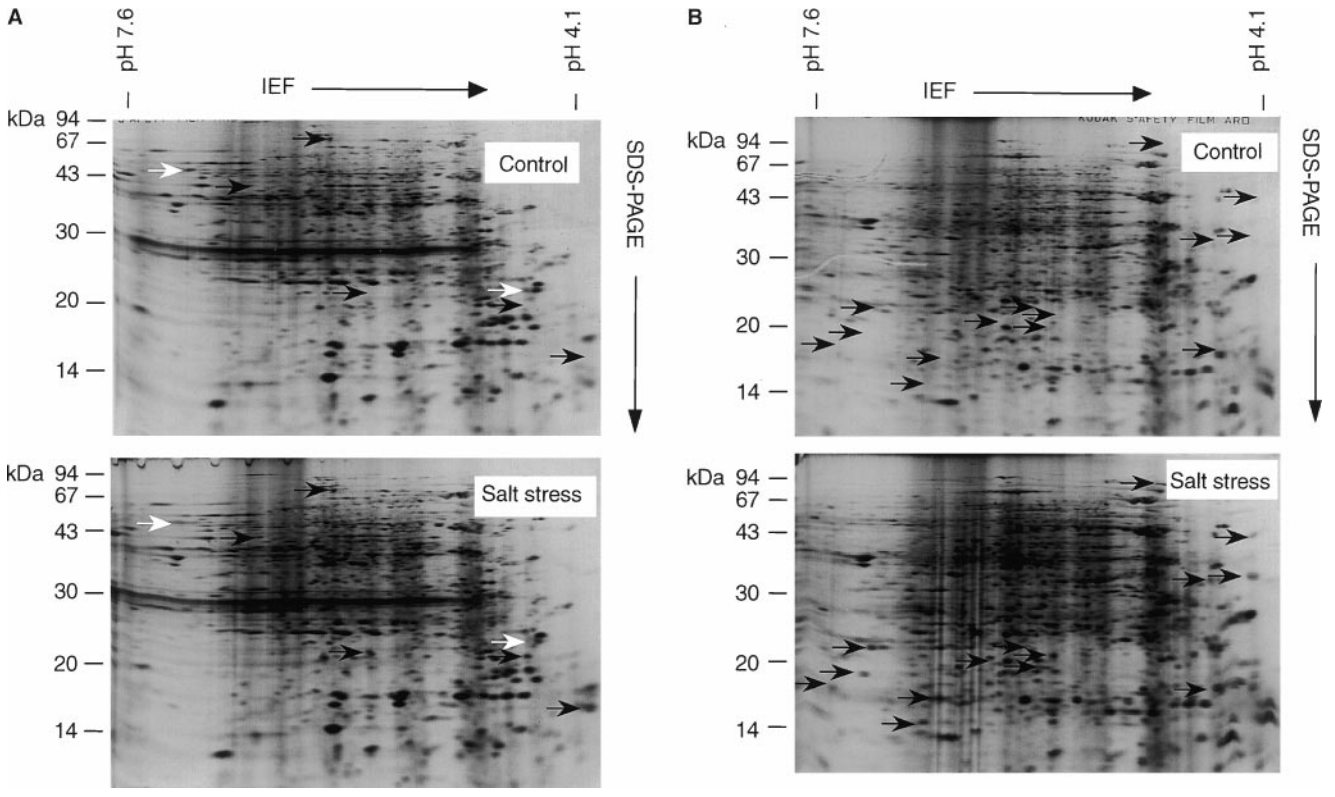

Fig. 1A, B Two-dimensional electrophoresis profiles of *in vitro* translation products of wheat leaf **A** and root **B** mRNAs. mRNAs from untreated (*Control*) seedlings and those treated with 0.15 M NaCl for 2 h (*Salt stress*) were translated *in vitro* in the presence of [^{35}S]methionine, and resolved by two-dimensional electrophoresis. *Black arrows* indicate polypeptides induced or enhanced by the salt treatment, while *white ones* show polypeptides decreased by salt treatment

apparent by 2 h, significant changes in gene expression in both leaves and roots were detected at this early stage.

Differential display

We used 27 and 26 primers for RT-PCR of leaf and root mRNAs, respectively. The number of amplified cDNA fragments varied between 10 and 42, ranging in size from 0.23 to 1.35 kbp, depending on the primers employed. The average numbers of the fragments were 19 from leaf cDNA and 18 from root cDNA. In total, we observed 490 fragments (Table 1). One example of the differential display patterns is shown in Fig. 2. We identified 32 and 29 differential fragments in leaf and root in repeated experiments, respectively. The differential fragments were excised from the gel and re-amplified by PCR with the same primer as used for the first reaction. The re-amplified PCR product size was checked to determine whether it was identical to the original one. Several fragments were not re-amplified, not showed the expected size.

Accumulation of transcripts after salt treatment

The re-amplified fragments, 16 from leaves and 11 from roots, were used as probes for the Northern hybridization of RNA from leaves or roots of 0.0 M or 0.15 M NaCl-treated wheat seedlings. The six probes from the leaf and the three from the root detected NaCl-treatment responding transcripts in Northern analysis, and these were then cloned by the TA system. The cloned cDNAs were again used as probes for Northern analysis to assess the response to NaCl treatment. Three clones from leaves and two from roots were confirmed to accumulate their transcripts after NaCl treatment.

Table 1 Number of differentially amplified fragments observed after the NaCl treatment

Organ	Total number of fragments appearing on the gels	Differentially amplified fragments		
		Induced or enhanced	Decreased or suppressed	Total
Leaf	486	25	7	32
Root	494	24	5	29

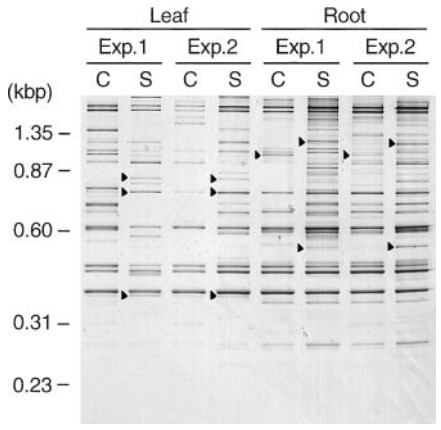

Fig. 2 A differential display pattern of cDNA fragments. The *arrowheads* indicate fragments that showed reproducible difference between the control (*C*) and the 0.15 M NaCl treatment (*S*) in two independent experiments (*Exp. 1*, *Exp. 2*)

We collectively named them WESR (Wheat Early Salt-stress Responding) genes.

We analyzed the effect of NaCl treatment for 2 h on the expression of the WESR genes by Northern analysis. Transcripts of WESR1, 2 and 5 were detected in 10 μg of total RNA on the blot (Fig. 3 A). The other two clones detected no signal on the blot of total RNA, and thus were hybridized with poly(A)$^+$ RNA (Fig. 3 B). We found that the accumulation of their transcripts were increased by NaCl treatment. Although WESR1 was derived from leaves, its transcript was detected more in roots than in leaves. Other transcripts were detected only in RNA of the original organs from which each cDNA clone had been derived. The loading amount of total RNA was standardized using rRNA (Fig. 3 A). The amount of transcript of the 0.15 M NaCl treatment, relative to that of the control, ranged from 1.3 to 2.6 (Table 2).

Sequence analysis of WESR genes

To examine the structure of WESR cDNA clones, we sequenced the cDNA fragments. Table 2 summarizes their characteristics. WESR1, WESR2, and WESR3 did not show any significant sequence similarity with known genes in databases. WESR2 showed 73.3% homology to one of the rice EST clones (Accession No. D15419, Sasaki et al. 1994) from a callus cDNA library, and to a rice matrix association region DNA (Accession No. X95271). WESR3 showed 70.2% homology to one *Arabidopsis thaliana* EST clone (Accession No. T22145, Newman et al. 1994). The deduced amino acid sequence of WESR4 showed 48.0% homology to that of a barley cDNA clone, ES43, which has the conserved DNA-binding domain sequence of the steroid hormone receptors (Speulman and Salamini 1995). The potential zinc-finger motif of WESR4 is defined by four

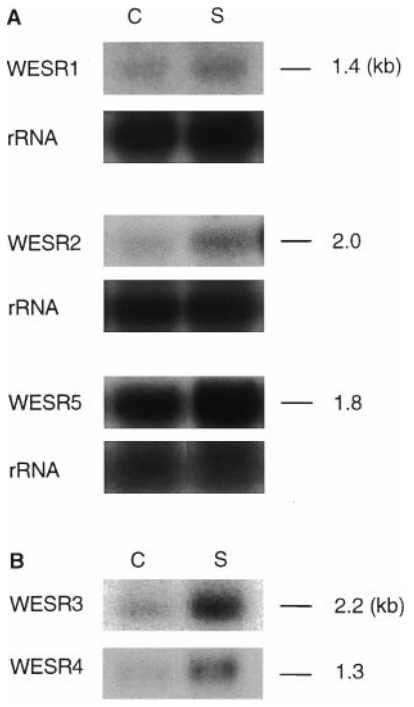

Fig. 3A, B Northern hybridization signals of salt stress-responding genes. Each lane contained 10 μg of total RNA **A**, or 4 μg of poly(A)$^+$ RNA **B**. The transcripts of WESR3 and 4 were detected only in poly(A)$^+$ RNA. Transcripts of WESR2 and 3 were detected in RNAs from leaves; and the others in RNAs from roots. All transcripts showed increased signal intensity after salt treatment. The size of the hybridizing mRNA was estimated based on RNA size markers. *C* control, *S* salt treatment

Table 2 cDNA clones from salt-responding genes from wheat seedlings

Clone	cDNA clone length (bp)	Size of transcript (kb)	Enhancement of transcript accumulation[a] (fold)
WESR1	567	1.4	1.3
WESR2	760	2.0	1.3
WESR3	341	1.9	2.6
WESR4	390	1.3	2.5
WESR5	743	1.8	1.4

[a] Enhancement of transcript accumulation was quantified by BAS2000 and based on a comparison with the control

cysteine metal ligands, arranged as a Cys-X_2-Cys-X_{23}-Cys-X_2-Cys (C_2-C_2) motif. The deduced amino acid sequence of WESR5 exhibited 80% identity to that of the glucose-6-phosphate dehydrogenases (G6PDH) from potato (Graeva et al. 1994) and alfalfa (Fahrendorf et al. 1995). The nucleotide sequence data reported in the present paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB011442 (WESR1), AB011443 (WESR2), AB011444 (WESR3), AB011445 (WESR4) and AB011441 (WESR5).

Discussion

In this study, we aimed to characterize the early events of the salt-stress response of wheat at the gene expression level by adding 0.15 M NaCl to liquid medium. We documented the change of gene expression both in leaves and roots after 2 h of treatment with 0.15 M NaCl by two-dimensional gel electrophoresis of the *in vitro* translation products. This indicates that wheat plants respond rapidly to NaCl treatment at the gene expression level. On the other hand, Gulick and Dvořák (1987) found no sign of a salt response of wheat leaves by a similar two-dimensional electrophoresis analysis of *in vitro* translation products after 6 hours of NaCl treatment. This disagreement may be due to the difference in the experimental design, including the treatment and the developmental stage of the plants. While Gulick and Dvořák (1987) transplanted 1-month-old wheat to NaCl solution, during which the concentration was gradually increased to 250 mM, we transferred 2-week-old seedlings suddenly to 150 mM NaCl solution. In both cases, more increased translation products were detected in roots than in leaves, probably because the roots have direct contact with the NaCl solution as suggested by Gulick and Dvořák (1987).

In the present experiment, we modified the simplified differential display method (Yoshida et al. 1994) by substituting a native acrylamide gel for the agarose gel in order to improve resolution, and employed the silver staining method instead of ethidium bromide to detect minor fragments and changes of gene expression. Each differential display experiment was repeated twice or three times to minimize false-positive fragments. The reproducible differences were further confirmed by Northern analysis. We were able to successfully identify the novel salt-stress responding genes at an early stage of stress, proving the effectiveness of the simplified differential display method to detect differences in extremely small quantities of gene products.

We characterized five cDNA clones that represented NaCl-treatment responding genes. WESR4 has a potential for encoding a protein with a zinc-finger motif, and WESR5 showed homology to G6PDH. Sequences of the other clones showed homology only to EST clones of rice (Sasaki et al. 1994) or *A. thaliana* (Newman et al. 1994), which are functionally unknown. All sequences homologous to the WESR genes have not been reported to respond to salt stress.

The deduced amino acid sequence of WESR4 was found to be homologous to that of the ES43 clone from barley (Speulman and Salamini 1995). ES43 was cloned with a pair of PCR primers designed from the amino acid sequence of the DNA-binding domain of the estrogen receptor. The homologous region between WESR4 and ES43 has a potential C_2-C_2 type of zinc-finger motif. Zinc-finger domains in proteins interact with nucleic acids to regulate gene expression in both prokaryotic and eukaryotic cells (reviewed by Berg 1990), implying that the WESR4 protein is a transcription factor which triggers the expression of genes involved in a late salt-response event.

WESR5 was found to be highly homologous to the G6PDH genes isolated from potato (Graeva et al. 1994) and alfalfa (Fahrendorf et al. 1995). The alignment of the deduced amino acid sequences revealed high homology at the C-terminal end which suggested that the amino acid sequences of this enzyme are highly conserved in plants. In alfalfa, it was reported that transcription rates for G6PDH were increased by elicitor treatment (Fahrendorf et al. 1995). G6PDH is known to catalyze the first step of the pentose-phosphate pathway (Copeland and Turner 1987), providing NADPH for anabolic metabolism. It would be interesting to determine if the pentose-phosphate pathway is activated by NaCl treatment, which might lead to an enhanced demand for reductants.

Several salt-stress responding genes have been isolated from stressed plants (Winicov 1994). In the case of ESI clones isolated from wheatgrass (Gulick and Dvořák 1990), the accumulation of mRNAs showed a biphasic response during the period of salt stress (Gulick and Dvořák 1992; Galvez et al. 1993). A similar response was also observed by the application of exogenous ABA. Thus, some genes induced by other types of stress than salt, such as drought, exposure to ABA or osmotic shock, are also related to the NaCl response (Shinozaki and Yamaguchi-Shinozaki 1997). Since salt stress is accompanied by osmotic stress, it is likely that the activation of these genes is controlled by many factors. Indeed, a WESR gene was also found to respond to ABA treatment (data not shown). Further characterization of the WESR genes in relation to other stress treatments would enhance our understanding of the salt responses in plants.

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