PERGAMON

Phytochemistry 61 (2002) 129–133

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Differential stress responses of early salt-stress responding genes in common wheat

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Received 29 November 2001; received in revised form 11 June 2002

Abstract

Four early salt-stress responding genes (WESR1–4) in common wheat (*Triticum aestivum* L.) were analyzed for their temporal accumulation of mRNA during salt stress, osmotic stress and abscisic acid (ABA) treatment. All genes showed transient stimulation by 0.15 M NaCl treatment. WESR1 and WESR2 were induced by both osmotic stress and exogenous ABA treatment. WESR3 responded to exogenous ABA, but not to osmotic stress. WESR4 did not show significant response to either osmotic stress or exogenous ABA treatment. These results suggest that wheat has at least two salt stress signal transduction pathways, an ABA-dependent and ABA-independent pathway.

Keywords: Abscisic acid (ABA); Osmotic stress; Salt stress; Semi-quantitative RT-PCR; Wheat

1. Introduction

Salt stress induces various biochemical and physiological responses in plants. It affects almost all plant functions, including photosynthesis, growth and development. 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). Under salt stress conditions, endogenous levels of a plant hormone, abscisic acid (ABA) increase (Gómez et al., 1988), which acts as a signal to promote tissue acclimation (Chandler and Robertson, 1994). Elevated ABA levels have been correlated with increased tolerance to salt (Singh et al., 1987), and exogenous application of ABA accelerated the adaptation of cultured tobacco cells to salt (LaRosa et al., 1987), which provide further support for a role of ABA in the acclimation of plants to osmotic stress. The correlation between osmotic stress and change in the ABA level has been well established at the molecular level (Shinozaki and Shinozaki-Yamaguchi, 1996).

Salt stress induces expression of a number of dehydration related genes, most of which are also responsive to ABA (Winicov, 1994). These genes are predicted to play a role in the adaptive response to stresses (Bohnert et al., 1995). In order to understand plant responses to salt stress, it is necessary to identify genes involved in the salt response and examine their characteristics in relation to other stress factors. We have previously isolated five wheat early salt-responding (WESR) genes at 2 h after exposure to 0.15 M NaCl to investigate the early events of the salt stress response at the level of gene expression (Nemoto et al., 1999). WESR1 and WESR2 showed sequence homology to EST clones of maize and rice, respectively, and WESR4 was predicted to encode a protein with a zinc-finger motif, but none of them showed homology to genes with known functions (Nemoto et al., 1999). Deduced amino acid sequence of WESR3 have sequence similarity with barley *Mlo* gene (Büschges et al., 1997), which is the first of a novel class of plant integral membrane proteins involved in fungal pathogen resistance. It has not been reported to respond to salt stress.

The WESR5 clone was shown to be homologous to glucose-6-phosphate dehydrogenase (G6PDH) gene. It

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0031-9422/02/$ - see front matter
PII: S0031-9422(02)00228-5

was demonstrated that transcriptional activation of the WESR5 (G6PDH) gene specifically responded to NaCl stress, but not to osmotic stress nor exogenous ABA treatment (Nemoto and Sasakuma, 2000).

In this study, we analyzed temporal gene expression of WESR1, WESR2, WESR3 and WESR4 under salt stress in relation to osmotic stress and exogenous ABA.

2. Results and discussion

Several salt-stress responding genes have been isolated from plants exposed to other stresses, such as drought and low-temperature (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 (Galvez et al., 1993). They also found a similar response of the genes following application of exogenous ABA. Thus, some genes induced by abiotic stresses other than salt, such as drought, exposure to ABA, or osmotic shock, can be related to responses to NaCl (Shinozaki and Yamaguchi-Shinozaki, 1997). Since salt stress is accompanied by osmotic stress, it is likely that activation of these genes is controlled by many factors. To determine whether osmotic stress or ABA is involved in the induction of WESR genes during salt stress, we performed quantification of their transcripts under different stresses by semi-quantitative RT-PCR analysis.

Semi-quantitative RT-PCR assay was performed by analyzing in parallel a gene whose expression was expected to be constant in most conditions. The α-tubulin gene has been widely used as a control in gene expression analysis under various physiological condition (Kawasaki et al., 2001; Seki et al., 2001) and was thus adopted as an internal control in this study. Amplification of wheat α-tubulin cDNAs indicated that approximately equal amounts of cDNAs were used for each RT-PCR analyses (Fig. 2). Quantification of transcripts were further confirmed by repeated RT-PCR analyses in which different RNA samples were used (data not shown).

The amount of PCR product increases exponentially in early cycles of the reaction, but subsequently reaches a plateau level. For each primer combination, PCR cycles where products do not yet reach a plateau level were determined as follows: During RT-PCR cycling, one sample was collected every two or three cycles of reaction, and subjected to Southern hybridization with a respective radiolabelled cDNA probe. On the basis of these experiments, all quantitative RT-PCR analyses were conducted at 18 cycles, when amplification has not reached a plateau level (data not shown).

To avoid the effect of genomic DNA-derived PCR products, we performed PCR with genomic DNA as templates, and compared the sizes of products derived from genomic DNA and expected size of cDNA fragments (Table 1). For all cases, sizes of PCR products amplified from genomic DNA were larger than those deduced from cDNA sequences, probably because of occurrence of introns, showing that quantities of RT-PCR products are not influenced by genomic DNA contaminating in RNA preparations. The transcript accumulation of WESR2 was analyzed by quantitative RT-PCR analysis, and confirmed that the result was equivalent to that of northern analysis (data not shown).

Total RNAs were isolated from roots of wheat seedlings after treatment with 0.15 M NaCl, as well as from the unstressed plants. The mRNA levels were determined by RT-PCR-based analysis, which showed that the levels of steady-state mRNAs of WESR 1–4 increased immediately after the salt stress treatment with peak at 2 or 12 h, and declined to basal level at 72 h (Fig. 1).

Since salt stress is accompanied by osmotic stress and triggers synthesis of ABA which induces the expression of many genes (Giraudat et al., 1994) the effect of osmotic pressure with 5.0% (w/v) mannitol and treatment with exogenous 20 μM ABA were also examined by semi-quantitative RT-PCR analysis. Total RNAs were isolated from roots stressed with 5.0% (w/v) mannitol or 20 μM ABA in comparison with the unstressed controls. The effect of ABA on transcript accumulation was analyzed at the time when each gene showed maximal expression level (2 h after ABA treatment for WESR1; 12 h for WESR2, WESR3 and WESR4: Fig. 2).

WESR1 and WESR2 transcripts accumulated in response to both osmotic stress and exogenous ABA treatment (Fig. 2). The WESR1 transcript accumulated 4.0-fold after 6 h osmotic stress treatment, and 2.8-fold after 2 h ABA treatment. The WESR2 transcript accumulated 1.8-fold after 2 h mannitol treatment, and 4.3-fold after 12 h ABA treatment. Many drought- or salt stress-inducible genes are also induced by exogenous ABA treatment. These genes have been shown to contain potential ABA-responsive elements (ABREs) in their promoter region (Bray, 1997). The results of the present study suggest that WESR1 and WESR2 gene expression is stimulated by salt stress through an ABA-dependent pathway which has been activated secondarily by salt stress treatment. These genes possibly contain ABA-responsive elements in promoter regions, although the possibility that they also have salt- and/or osmotic stress-responsive elements cannot be excluded. The WESR3 transcript did not respond to osmotic stress, but did respond to ABA treatment. The WESR3 gene was induced by exogenous ABA treatment, but was not induced by osmotic stress, suggesting that the gene has an ABA-responsive element, but not an osmotic stress responsive element. The WESR4 gene did not show significant response to either osmotic stress or exogenous ABA treatment.

Based on the pattern of mRNA accumulation, it is hypothesized that at least three independent signal

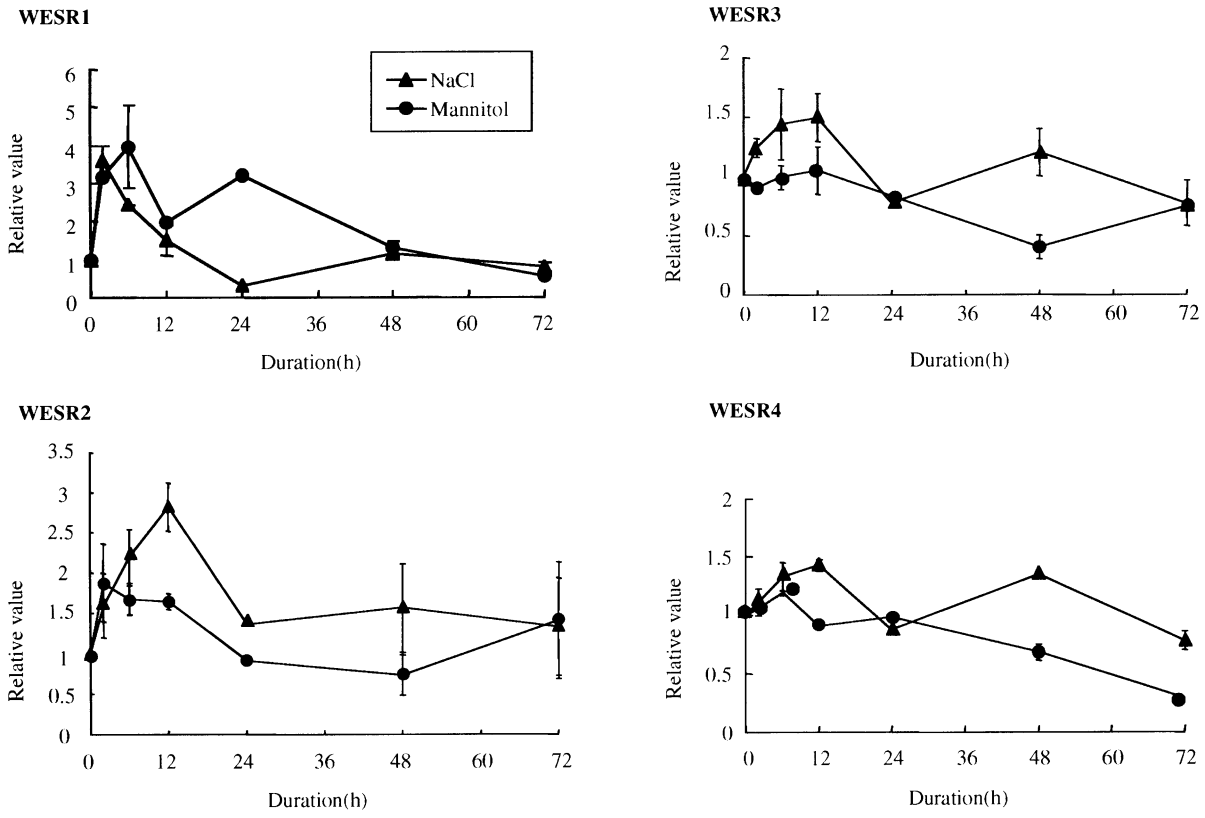

Fig. 1. Temporal accumulation of WESR gene transcripts under salt stress and mannitol treatment. Accumulation of WESR gene transcripts in root were quantified by RT-PCR followed by Southern hybridization. Hybridization signals of WESR genes were standardized to those of α-tubulin gene. Normalized values for WESR gene transcripts from stress-treated plants were divided by those from unstressed plants (control) at respective time to cancel the effect of medium exchange at 0 h, and plotted as relative value against time. Standard errors are indicated. Values represent means±s.e. of two or three replicates.

Table 1
Primer used in PCR and RT-PCR analyses

Primer name	Sequence	Size of PCR product	
		cDNA (bp)	Genomic DNA (kb)
WESR1			
WR1B	5-GGCGAACATTCGTCGGCTATCTCA-3	355	1.4
WR1F	5-GTACTGTACATGCCCTAGGAATCA-3		
WESR2			
WR2A	5-ATCAGCACACGCAAAGAAATG-3	362	0.6
WR2D	5-CGATGGTATGTGGTTGCAGA-3		
WESR3			
WR3A	5-CTTGTGGGTCTTCGTGGTGAT-3	244	0.7
WR3D	5-ATGGATCAGGAACAGGACGA-3		
WESR4			
WR4A	5-GGTGCAATGCGAGGGATGCAAAGA-3	256	2.7
WR4C	5-GCAATGGAGGGCTGACGCCATAAA-3		
α-Tubulin			
Ta α-tubulin 2	5-ACCGCCAGCTCTTCCACCCT-3	579	0.7
Ta α-tubulin 3	5-TCACTGGGGCATAGGAGGAA-3		

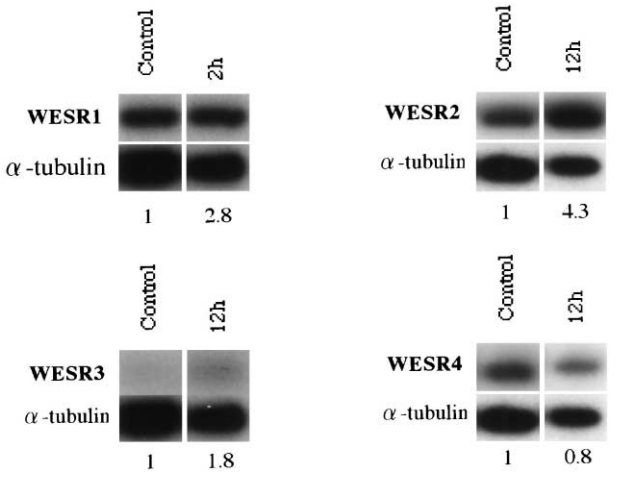

Fig. 2. Effects of exogenous ABA treatment on accumulation of WESR gene transcripts. Accumulation of WESR gene transcripts were quantified by RT-PCR followed by Southern hybridization. Signals of WESR genes and α-tubulin genes were presented for stressed and unstressed (control) plants. Quantitative data for effect of ABA were obtained at the time when enhancement of transcription accumulation reached the peak by salt treatment (Fig. 1). Values represent means of two replicates.

pathways function in the activation of salt stress-inducible genes: two are ABA-dependent (pathway II: respond to both ABA and osmotic stress, III: respond to ABA but not to osmotic stress) and one is an ABA-independent (pathway I). The WESR1 and WESR2 are categorized in pathway II, WESR3 is in pathway III, and WESR4 is in pathway I.

The present study suggests the existence of several ABA-independent and ABA-dependent signal transduction pathways between the initial perception of salt stress signal and expression of specific genes in wheat, as well as *Arabidopsis* (Shinozaki and Shinozaki-Yamaguchi, 1996). We have previously found that the WESR5, which encodes glucose-6-phosphate dehydrogenase (G6PDH), did not respond to either osmotic stress or exogenous ABA (Nemoto and Sasakuma, 2000). So far, WESR4 and WESR5 are the only genes which showed specific response to salt stress in wheat. Further identification of salt-stress responding genes and analysis of gene inducing factors will be required for understanding events in salt stress responses in plants.

3. Experimental

3.1. Plant material and stress treatments

Wheat plants (*Triticum aestivum* L. cv. Chinese Spring) were grown hydroponically in 1/5 strength Linsmaier–Skoog (LS) medium (Linsmaier and Skoog, 1965) in a growth chamber with continuous illumination under the conditions described previously (Nemoto et al., 1999). Two-week-old seedlings were transferred to fresh LS medium supplemented with 0.15 M NaCl, or 5.0% (w/v) mannitol or 20 μM abscisic acid (ABA). The osmotic potential of 5.0% (w/v) mannitol was evaluated to be equivalent to that of 0.15 M NaCl by measurement with an osmometer (OM-801, Vogel, Germany).

3.2. Semi-quantitative RT-PCR analysis

Total RNAs were isolated from the roots of the seedlings that were exposed to 0.15 M NaCl, 5.0% (w/v) mannitol, or 20 μM ABA as described previously (Nemoto et al., 1999). Normalized values for WESR gene transcripts from stressed plants were divided by those from unstressed plants (control) at respective time to cancel the effect of medium exchange at 0 h.

The RNA samples were denatured for 10 min at 70 °C and cooled on ice. Reverse transcription was carried out in a final reaction mixture (100 μl) containing first strand buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 6 mM dithiothreitol, 0.3 mM dNTPs, 2 ng of oligo $d(T)_{20}$ and 30 units of Superscript™ II RNase H^- reverse transcriptase (Gibco-BRL). The reactions were performed at 42 °C for 60 min. The mixture was then heated to inactivate the enzyme at 70 °C for 15 min, and the samples were either used directly for PCR or stored at −20 °C.

RT-PCR was performed using 4 μl of each cDNA sample in a final reaction mixture (20 μl) containing PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.1 mM dNTPs, 1 μM of each forward and reverse primers and 0.25 units of *Taq* polymerase (Takara Shuzo). The primer sequences used and predicted size of amplification products are indicated in Table 1. Fragment of α-tubulin gene (accession No. U76558) was amplified with specific primers (Table 1) as a control for semi-quantitative RT-PCR. The PCR was performed for 18 cycles with the following temperature profile: denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C or 62 °C, and extension for 30 s at 72 °C. Eight microlitres of PCR products were resolved on a 2% agarose gel and blotted onto a nylon membrane (Hybond-N^+, Amersham Pharmacia biotech) with 0.4 N NaOH for 2 h. cDNA clones for WESR1, WESR3, WESR4 and α-tubulin were labelled with [α-^{32}P]dCTP and used as probes. Hybridization was performed at 68 °C overnight with the hybridization buffer containing 6×SSC, 5×Denhardt's solution, 0.5% SDS and 10 μg/ml denatured salmon sperm DNA (Sambrook et al., 1989). After hybridization, the membrane was washed twice at 68 °C with 0.1×SSC/0.1% (w/v) SDS for 20 min. Quantitative data were obtained from the hybridization signal by use of BAS-2000 (Fujix) and BAStation software to sum total pixel values in equal-size areas encompassing signals. Hybridization signals of WESR

genes were standardized to that of α-tubulin gene. Normalized values for WESR gene transcripts from salt-stressed plants were divided by those from unstressed plants (control) at respective time to cancel the effect of medium exchange at 0 h.

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