Gene 529 (2013) 321–325

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short Communication

Salicylic acid increases the contents of glutathione and ascorbate and temporally regulates the related gene expression in salt-stressed wheat seedlings

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ARTICLE INFO

Article history:
Accepted 29 July 2013
Available online 13 August 2013

Keywords:
Ascorbate
Glutathione
Salicylic acid
Salt stress
Wheat

ABSTRACT

Exogenous salicylic acid (SA) significantly improved abiotic tolerance in higher plants, and ascorbate (ASA) and glutathione (GSH) play important roles in abiotic tolerance. In this study, SA (0.5 mM) markedly increased the contents of ASA and GSH in SA-treated plants during salt stress (250 mM NaCl). The transcript levels of the genes encoding ASA and GSH cycle enzymes were measured using quantitative real-time PCR. The results indicated that, during salt stress, exogenous SA significantly enhanced the transcripts of glutathione peroxidase (*GPX1*), phospholipid hydroperoxide glutathione peroxidase (*GPX2*) and dehydroascorbate reductase (*DHAR*) genes at 12 h, glutathione reductase (*GR*) at 24 h, 48 h and 72 h, glutathione-S-transferase 1 (*GST1*), 2 (*GST2*), monodehydroascorbate reductase (*MDHAR*) and glutathione synthetase (*GS*) at the 48 h and 72 h after salt stress, respectively. The results implied that SA temporally regulated the transcript levels of the genes encoding ASA–GSH cycle enzymes, resulting in the increased contents of GSH and ASA and enhanced salt tolerance.

1. Introduction

Salt stress is one of the primary limiting factors in agricultural crop production. It causes water deficit, ion toxicity, and nutrient deficiency, leading to growth and yield reduction, and even to plant death. About 7% of the world's land is affected by salt stress, which seriously impairs crop production at least 20% of irrigated land worldwide (Munns and Tester, 2008). Wheat is an important cereal crop and is a salt-sensitive glycophyte. The growth and grain yield of wheat are significantly affected by soil salinity. Some literatures have recently been published on wheat salt-responsive proteins or the molecular mechanisms of salt tolerance in wheat (Caruso et al., 2008; Gao et al., 2011; Mott and Wang, 2007).

Salicylic acid (SA) is an important signalling molecule that has an essential role in plant defence responses induced by various pathogens (Snyman and Cronjé, 2008). Recent reports have demonstrated that SA also has important roles in modulating the plant responses to many abiotic stresses such as salt, drought, chilling, and heat. However, abiotic tolerance induced by SA is poorly understood, and studies on SA-modulated abiotic tolerance have mainly been performed at the physiological levels. These studies suggest that SA might have acted as an antioxidant for scavenging reactive oxygen species (ROS) generated under the abiotic stress conditions (Horváth et al., 2007).

Enzymatic antioxidant systems [e.g. superoxide dismutases (SOD), catalase (CAT), and peroxidase (POD)], and non-enzymatic compounds, such as ASA and GSH, are involved in defence against ROS induced by abiotic stress (Carillo et al., 2008, 2011; Mittler, 2002). In previous studies, SA remarkably increased ASA and GSH contents and transcripts of the genes encoding ASA–GSH cycle enzymes in cold-stressed rice and eggplants, suggesting that ASA–GSH biosynthesis has an important role in cold tolerance induced by SA (Chen et al., 2011; Kang and Saltveit, 2002). Different plant species show differential physiological responses to different biotic or abiotic stressors (Wang et al., 2010). In mungbean (*Vigna radiata* L.), the application of 0.5 mM SA remarkably increased activity of antioxidant enzymes and contents of GSH, photosynthesis under salt stress condition (Khan et al., 2010; Syeed et al., 2011). However, to our knowledge, no investigation has been reported on the relationship between SA and ASA–GSH biosynthesis under salt stress conditions in higher plants at molecular level. In the present study, we measured the transcript levels of the genes encoding ASA–GSH cycle enzymes in SA-treated wheat seedlings under salt stress in order to explore the relations between SA-induced salt tolerance and ASA–GSH biosynthesis in higher plants at molecular level.

Abbreviations: ASA, ascorbate; CAT, catalase; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GR, glutathione reductase; GS, glutathione synthetase; GSH, glutathione; GST, glutathione-S-transferase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; POD, peroxidase; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutases.

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http://dx.doi.org/10.1016/j.gene.2013.07.093

2. Materials and methods

2.1. Plant materials

Seeds of common wheat (*Triticum aestivum* L. cv. "Yumai 34") were sterilised with 0.01% $HgCl_2$. Sterilised seeds were grown hydroponically in full-strength Hoagland solution in glass dishes (15 cm diameter) in a FPG-300C-30D illumination incubator (Ningbo Laifu Technology Co., Ltd., China) at a 14-h photoperiod, 25 °C/15 °C day/night temperatures, light intensity of 250 μmol m^{-2} s^{-1}, and relative humidity of 60%/75% (day/night). Two-week-old seedlings with two fully expanded leaves were used in this study.

2.2. Salt stress

Salt stress was imposed as described previously (Kang et al., 2012). Briefly, wheat seedlings were incubated with fresh Hoagland medium supplemented with 250 mM NaCl solution for salt stress, and other seedlings were transferred to Hoagland solution supplemented with 250 mM NaCl solution and 0.5 mM SA (NaCl and SA treatment, salt + SA). Seedlings grown under the same conditions without NaCl or SA treatments were used as control (CK). The uppermost fully expanded leaves were harvested from each treatment at 0 h, 12 h, 24 h, 48 h and 72 h after salt stress; a portion of fresh leaves were used to measure contents of ASA and GSH. The remaining leaves were immediately frozen in liquid nitrogen and stored at −80 °C.

2.3. Assays of ASA and GSH contents

The contents of total ASA and GSH were measured according to the methods of Kampfenkel et al. (1995) and Smith (1985), respectively.

2.4. Transcript levels of the eight genes encoding ASA–GSH cycle enzymes using quantitative real-time PCR (qPCR) in salt-stressed wheat seedlings

The expression patterns of the eight genes encoding ASA–GSH cycle enzymes were measured in cold-stressed eggplant seedlings (Chen et al., 2011). They include *GST1*, *GST2*, *GPX1*, *GPX2*, *GR*, *DHAR*, *MDHAR*, and *GS*. In this study, the expression patterns of these eight genes were also measured in wheat seedlings during salt stress condition according to the methods of Chen et al. (2011).

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction and treated with RNase-free DNase I (Takara Biotechnology [Dalian] Co., Ltd., Dalian, China) to remove contaminating genomic DNA. First-strand cDNAs were synthesised from 2 μg of total RNA using Super-Script II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qPCR is performed using a SYBR Premix Ex Taq (Perfect Real Time) kit (Takara Biotechnology [Dalian] Co., Ltd.) on a Light Cycler 480 machine (Roche Diagnostics) according to the manufacturer's instructions. Specific primers for each gene were designed (Table 1). The real-time PCR amplification mixture (50 μl) contained 1 μl of cDNA, 20 μl of QuantiTect SYBR Green PCR Master Mix, and 2 μl of forward and reverse primers (10 μM). Triplicate quantitative assays were performed on each cDNA sample. The wheat *Actin* gene (GenBank accession no: AB181991) was used as an endogenous control. Each data point was expressed as the average ± SD of three independent replicates.

2.5. Statistical analysis

All experiments were repeated three times with sixty plants in each replication. Data were subjected to ANOVA analysis by SPSS (version 17.0) and Duncan's multiple range tests ($P < 0.05$) to compare the mean value of different treatments. Each data point was expressed as the average ± SD of three independent replicates.

Table 1
DNA sequences of PCR primers were used for qPCR determination of the eight ASA–GSH biosynthesis-related genes in wheat seedlings. F, forward primer; R, reverse primer.

Gene	Accession no.	Primer pairs	Expected amplification size (bp)
GST1	JX051003	F: GACGAGGCGTGGAAGGACGGCT R: GGGAAGATGGCGGCGTTGC	113
GST2	JX051004	F: AGCTCTTGGCGTCTTGGCT R: AGGCTTCCCCTTGGAGCAC	131
GPX1	AF475124	F: CTCGCTTCAAGGCTGAGTA R: CCACCTTTGCTAGACTTCAG	97
GPX2	JN578723	F: CCTAACTAACTCCAACTACACC R: TCCTGCCCACCAAACTGAT	105
GR	AY364467	F: ATGAATACTCCCGTACATCAGT R: TTTGTTACATCACCCACAGC	55
DHAR	AY074784	F: GTGCCTGTGTATAACGGTG R: ACAAGTGATGGAGTTGGGT	94
MDHAR	AK371371	F: AGAAGTTTACGCCCTTCGGC R: TTGGAATGTCATCGCCATC	132
GS	AJ579382	F: ATCGCCAAGCTCCGTCAATG R: ACAAGTCAGGGTTTTCAATCG	88
Actin	AB181991	F: AGCGGTCGAACAACTGGTA R: AAACGAAGGATAGCATGAGGAAGC	101

3. Results and discussion

Salt stress leads to slow growth, wilting or even death of plants, and oxidative stress caused by salt stress may be one of the primary causes of severely disrupted protein synthesis and acts by interfering with normal enzyme activity. The accumulation of ROS causes lipid peroxidation and protein degradation, even cell death. To alleviate the effects of ROS, both enzymatic and non-enzymatic antioxidants have evolved in plants. In our and other previous studies, exogenous SA application significantly elevated levels of endogenous SA, decreased concentration of lipid peroxidation (MDA), improved the activities of antioxidant enzymes and contents of non-enzymatic compounds, improved the ratio of K^+/Na^+, and increased the growth of plant (e.g. the heights, fresh and dry weights), resulting in the enhanced abiotic tolerance (Chen et al., 2011; He and Zhu, 2008; Kang et al., 2012; Krajnc et al., 2011).

The ASA and GSH are the major non-enzymatic antioxidants, and the enzymes and antioxidants in the ASA–GSH cycle play important roles in scavenging ROS (such as H_2O_2) (Liu et al., 2012; Shan and Liang, 2010). Contents of GSH and ASA in salt-tolerant plant varieties are significantly higher than those in salt-sensitive varieties (Vaidyanathan et al., 2003). Overexpression of the genes encoding ASA–GSH cycle enzymes in higher plants conferred enhanced tolerance to abiotic stresses (e.g. salt, chilling) by maintaining higher contents of GSH and ASA (Duan et al., 2012; Eltayeb et al., 2007; Sultana et al., 2012). In the present study, the application of exogenous SA markedly raised the contents of both GSH and ASA in the leaves of wheat seedlings during salt stress (Fig. 1). After 72 h of salt stress, the contents of GSH and ASA in the leaves of plants treated with salt + SA were 33.1% and 44.0% higher than those treated with salt alone, respectively. This implies that the salt tolerance induced by SA in wheat seedlings may be related to increased contents of GSH and ASA.

It has been reported that there may be no transcriptional, post-transcriptional, translational, or post-transcriptional regulation of the genes encoding ASA–GSH cycle enzymes (Chen et al., 2011; Liu et al., 2012; Shan and Liang, 2010). Transcription analysis has the advantage of quantifying the changes in transcript levels of genes. Therefore, transcription analysis of genes could lay the foundation for the identification of genes involved in the regulation of metabolism and provide valuable insights into the molecular mechanisms of many biosynthetic pathways (Ohdan et al., 2005). In this study, the eight genes encoding ASA–GSH cycle enzymes were measured in salt-stressed wheat seedlings to explore the influence of SA on the ASA–GSH cycle at molecular level.