Resistance of wheat to mycosphaerella graminicola

Resistance of wheat to *Mycosphaerella graminicola* involves early of gene expression

INTRODUCTION

Wheat is one of the three cereal grain crops, together with rice and maize, being used for human food feed for more than 5000 years ago (Peng et al., 2011). The world annual wheat production is about 671 million tons in the total cultivated area (FAO, 2012). It is estimated that the demand for wheat will increase to 60% by 2050. However pathogenic and non-pathogenic stress factors can cause wheat production loss by 29% (Manickavelu et al., 2012). However, new molecular genetic methods and plant gene sequence data have focused on improving tolerant cultivars against biotic and abiotic environmental stress.

Zymoseptoriatritici blotch (STB) of wheat (Triticum aestivum L.), caused by Mycosphaerella graminicola (anamorph: Zymoseptoria tritici), occurs in all wheat growing areas worldwide, with an increasing major economic impact over the last ages [1]. The causal agent, S. tritici, was first described by Desmazières [2]. Sanderson identified the ascomycete M. graminicola as the sexual stage (teleomorph) of S. tritici [3, 4]. The origin of M. graminicola is most likely the Middle East [5]. Presently the fungus represents a major economic concern for global wheat production [8]. M. graminicola is developing rapidly as a model for fungi in the order Dothideales [6, 7].

Z. tritici is controlled by the use of resistant and tolerant cultivars and fungicides. However, current cultivars provide partial protection due to fast changes of pathogen populations in the field [4–6]. Moreover, environmental

concerns have increased limitation on the use of fungicides. Thus, more production of improved cultivars for controlling the disease is required. The development of new strategies for disease managing based on a plant's defense mechanisms can obtain suitable plant crops and improvement of human and environmental health for.

PCR (Polymerase chain reaction) techniques that record fluorescence in real time when samples pass photo detection diodes have been described. The sensitivity of this technique is similar or better than other PCR methods [2]. PCR-based real-time quantitative assays have been developed for the detection of a variety of plant pathogens [9–11], enabling high throughput detection of plant pathogen infection in a relatively short time.

To identify the resistance mechanisms, genes and genetic pathways underlying the STB in wheat transcriptomics analyses were undertaken, looking at differential gene expression in wheat leaf tissue at defined time points after *Z. tritici* . Leaf tissue was sampled at five time points after inoculation, including time points that represented the early stages of the pathogen's development. The transcript profiles of 8 selected genes were measured over the five time points by quantitative PCR (qPCR) and reverse northern blot.

Plant hormones play a important role in defense against pytopathogens, and salicylic acid, jasmonic acid, and ethylene are known as key players (Koornneef & Pieterse 2008). SA is always associated with resistance against biotrophic and hemibiotrophic pathogens, and with start systemic acquired resistance (SAR) in many species (Glazebrook 2005). SAR done protection

against a broad range of pathogens. Induction of SAR by SA is accompanied by the expression of a set of genes encoding pathogenesis related (PR) proteins and defense proteins in plants, such as wheat and *A. thaliana* (Ukneset al. 1992).

The induction of specific PR-proteins in plant species correlates with the onset of systemic acquired resistance (SAR)[47, 56] and can be induced by exogenous treatments of SA and other related chemical inducers[15, 59].

These stress-responsive genes are regulated by multiple signaling molecules [2, 3] with significant overlap between the patterns of gene expression that are induced in response to different stresses [4–6]. However, induced defense mechanisms can be costly to the plant, diverting resources that would otherwise be utilized toward reproduction [7, 8]. Finally, resistance mechanisms that prioritize the involvement of specific members of a gene family against the stress at hand, may result in less costly modes of defense.

Considerable advancement has been made in understanding events in the interaction of *M. graminicola* and wheat. Contact between the fungus and a living mesophyll cell is required for transfer of signals from the pathogen to the host cell and the induction of resistance[11].

The expression profiles during the first 4 days after inoculation (DAI), Ray et al. [29] indicated that protein disulfide isomerase (PDI) and the three PR proteins PR-1, PR-2 and PR-5, were induced in inoculated wheat leaves within 3–12h following contact with the pathogen. These 4 genes were induced much more strongly in the resistant cultivar Tadinia (containing the Stb4 gene) compared to the susceptible control Yecora Rojo, indicating that they

may contribute to defense responses. Precisely how these genes participate in the defense response in wheat is not clear, but a variety of roles has been proposed [30]. One possibility is that PR proteins inhibit the germination of spores and growth of the pathogens due to production of toxic intermediates[31]. The PDI and three PR proteins induction in the Stb4 defense gene peaked at from 6 to12h after inoculation, and the genes expression were similar to those in the susceptible cultivars by the last time point sampled, at 96 h after pathogen inoculation. These results showed that the outcome of the host pathogen interaction was determined by events occurring during the first 24h (Ray et al.)[29]. The latent period of this disease usually is more than 18 days. Whether the pathogen is killed after 24h and whether the plants can respond after the first 4 days after contact with the pathogen are not known. In addition to PR proteins and PDI, other genes that were up-regulated in the resistant cultivar Tadinia during the first 24h after inoculation were identified in a large-scale gene-expression-profiling experiment [32]. Although some PR proteins also responded, many of the genes that were induced by the pathogen had not been implicated in defense responses previously or had no useful annotations.

These could indicate novel types of resistance responses of wheat to M. graminicola. Whether these genes are associated with resistance responses of other genes besides Stb4in Tadinia, or if they show a different timing of gene expression relative to the PR proteins and PDI analyzed previously are not known. The purpose of this study was to analyze the expression patterns over time of some of the genes identified in previous expression-profiling experiments to test whether they are associated with the resistance

response. A secondary goal was to test whether the resistance response to M. Graminicola seen with theStb4gene extends to a gene on another chromosome and also at later time points in the infection process.

To accomplish this, we examined the levels of expression of 14 defense-related genes in the two resistant cultivars Tadinia (Stb4 gene on chromosome 7DS) and W7984 (Stb8gene on chromosome 7BL) from 0 to 27 DAI with M. graminicola. The 14 genes were chosen because they were among the most highly up-regulated in ARTICLE IN PRESS T. B. Adhikari et al. / Physiological and Molecular Plant Pathology 71 (2007) 55–68 56 preliminary analyses of resistance responses toM. grami-nicolain Tadinia. The final goal was to determine whether gene expression at 12h after inoculation could be used to separate resistant from susceptible plants as a rapid and accurate method of phenotyping.

Induced resistance to plant pathogens, for example the SAR response, can be obtained by using defense signaling compounds that activate the defense signaling pathways. (12, 17). The treatment of plants with jasmonates, such as SA, can enhance resistance in a wide range of plant pathogen interactions [17]. Research on SA has been extended further with the development of synthetic SA analogues such as 2, 6-dichlor-oisonicotinic acid (INA) and benzo(1, 2, 3)thiadiazole-7-car-bothionic acidS-methyl ester (BTH), which are more potent inducers of SA inducible plant defences [15, 35]. In wheat, BTH induced a specific set of genes termed wheat chemically induced (WCI) genes, more strongly than either INA or SA.

In this research, we have used real-time quantitative PCR (RT- qPCR) and reverse northern blot to measure expression of wheat defense genes. This has been applied to study defense gene activation following inoculation of tolerant wheat cultivar (Zagros). We have also studied the effects of SA on wheat defense gene expression disease development.

The data suggest that MJ is a more potent inducer of defence gene expression than BTH, and MJ treatment prior to inoculation significantly delays CR-lesion development in a genotype-independent manner.

MATERIAL AND METHODS

Plant materials and growth conditions

Plants of three *Triticum aestivum* cv. *Zagros* was grown in climatically controlled chambers under 16: 8 h day-night cycles at 22-18 ºC. Plants were watered as needed and fertilized weekly with 20-20-20 (N-P-K). Zagros is tolerant cultivar to ZTB.

The plants grown in 15 cm diameter pots filled with pre-fertilized soil and with five seedlings in each pot. Seedlings were grown to the two-leaf stage. All plants were grown in a greenhouse at 18-23 ºC with a 16-h photoperiod. Each experiment for each time point consisted of three replications.

Fungal inoculum and inoculation

The pathogen isolate was received from the Plant Pathology department of Tarbiat Modares University of Iran. The pathogen isolate (S1) was maintained on yeast malt extract agar (YMDA) at 4°C. Spores were scraped from the

surface of the cultures into a 0. 5% Tween 20 sterile distilled water solution and filtered through sterile cheesecloth before quantification using a haemocytometer. Spore concentration was adjusted to 10^{7} ml $^{-1}$. The wheat seeds were surface-disinfected with 70% alcohol for 5 seconds and it was washed twice with sterile distilled water. Then the seeds were sown in pots. The seedlings were grown in a greenhouse. The 12 days old seedlings (two leaf stage) grow in was sprayed with two concentrations of SA (0 and 2mM). Each pot was sprayed with 50 ml of the solutions (He and Wolyn 2005). After 24h of SA incubation, the plants were inoculated with a suspension of spores of *M. graminicola* . The control treatment was sprayed with sterile distilled water.

Sampling procedures

The treatments consisted of mock inoculation with water (control), inoculation with SA (2Mm), inoculation with *M. graminicola* and inoculation with SA and *M. graminicola,* jointly. For the time course infection assays, each treatment was sampled at 5 time points following inoculation (0, 3, 6, 12h and 24HAI), generating 20 samples in total (times 4 time points times four treatments). Plant Samples were frozen immediately after sampling and in this state they were ground to a fine powder using a mortar and pestle. Four plants for each sample were harvested, placed in a labeled plastic bag and stored at -80 ºC. A complete random design with four replications was used for the analysis of each RIL population. Each pot contained three plants, and three inoculated plant was considered as one replicate.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of leaf tissue using a CinnaPure-RNA Kit (Cinnagene, Iran), according to the manufacturer’s instructions. Extracted RNA was treated with DNase using a Qiagen RNase-Free DNase Set (Qiagen, Valencia, CA). Total RNA was visualized on a 1. 6% agarose gel and then quantified and assessed for purity using a UV–visible spectrophotometer (CECIL 9500 Model, UK). Sample purity was assessed using the ratio of absorbances at the 260 nm and 280 nm (A260/A280) wavelengths. Samples having A260/A280 more than 1. 8 and less than 2 were considered acceptable for use in quantitative real-time polymerase chain reactions (qPCR) and reverse northern blot assays. Total RNA was quantified based on the standard conversion factor of 1 absorbance unit at 260 nm = 40 mg RNA mL1 (Tsai et al., 2004).

The bioinformatics research and previous studies was used to select target genes that play a important role in wheat health through defense responses (Supplemental Table S2). Actin (Act), β- tubollin and Etf1a () genes were used as internal standards for qPCR normalization. Gene sequences were obtained through GenBank (2012) and qPCR primers (Supplemental Table S2) were designed using Oligo5 (Untergasser et al., 2012). Primers had a Tm of 59 C ± 2C and the amplification efficiency of each primer set was determined by creating a cDNA standard curve consisting of five dilutions of concentrated cDNA. Dilutions were subjected to qPCR and efficiency (E) was calculated using the equation $E = 10^{[-1/m]}$ where m is the slope of the standard curve (Pfaffl, 2001).

For cDNA synthesis, First, 1 Mg of total RNA was converted to cDNA using the RevertAid Reverse Transcriptase Kit (Thermo Scientific, Germany).

Conversions were carried out according to the manufacturer’s instructions in a total volume of 20 mL. Reverse transcription was conducted in a thermal cycler at 42 ºC for 1 h followed by 5 min. at 70 ºC to stop the reaction. Samples of cDNA was stored at -20 ºC.

Reverse Northern blot

After the genes were selected, the reaction PCR conditions were optimized for each of these genes, and they were reproduced by thermocycler set () model A. Aliquots of the amplified genes (200µL) were blotted in duplicate onto two Hybond-N + nylon membranes. The membranes were then hybridized, twenty to a total cDNA probe from 20 treatments of wheat. So, totally 20 probes were constructed for comparison between two concentration of SA and Presence or absence of fungal pathogen. α-Tubulin was used as a control blot in membranes.

Reveres Northern analysis

Hybridization was carried out overnight at 65ºC in 1mM NaH2PO4, 0. 5 M EDTA, 10 % SDS. The membrane was washed twice for 15 min in 2× SSC/0. 1 SDS at room temperature and then twice for 15 min each in 2×SSC/0. 1 SDS at 65 ºC. Probes was labeled by adding 1 mM of digoxigenin-dUTP and using the same PCR program and primers as for the Cdnasynthesis. Chemiluminescent detection of the Dig-Labeled nucleic acids was made with CDP-STAR according to the manufacturer’s instructions (Boehringer Mannheim). Before evaluate the relative abundance of the expressed gene transcripts, turbidity of the dots was normalized with α-tubulin using Total

Lab software. Finally, data were computed and converted into histograms with Microsoft Excel software.

Real-time quantitative PCR gene expression analysis

RT-qPCR was performed in an optical 384-well plate using an Corbett RG-6000 Sequence Detection System. Each reaction contained 10µl of YTA SYBR Green qPCR Master Mix (Yekta Tajhiz Azma, Iran), 0. 4 µM of both forward and reverse gene specific primers, listed in Table 1, and 50 ng cDNA. The following thermal profile was used for all PCRs: 95ºC for 10 min; 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Data were evaluated using Rotor-Gene 6000 Software and Microsoft Excel. Exponential amplification was plotted on a logarithmic scale and the Rn was set to 0. 24 for each RT-qPCR plate to obtain the cycle threshold (Ct) values. Ct values for all genes was normalized to TEF-1α of wheat to calculate for variation between PCR runs or different cDNA samples. In addition, control samples were always run on the same plate as their respective treated samples. Primer efficiencies were determined using the LinRegPCR program[32]. Student’s t-test was used to show significant differences between basal gene abundances in Kennedy and Sunco.