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Analysis of defense gene expression changes in susceptible and tolerant cultivars of maize (*Zea mays*) upon *Meloidogyne arenaria* infection

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ABSTRACT

Maize (*Zea mays*) is a major crop that is cultivated worldwide. It is susceptible to many pathogens, including root-knot nematodes (RKN, *Meloidogyne* spp.). RKN have a wide host range that includes many monocotyledons and dicotyledons. Infection with RKN induces changes in gene expression of pathogenesis-related (PR) and other defense-related proteins. We examined the expression levels of genes encoding selected maize proteins that are involved in the early stages of plant responses to *M. arenaria* infection, including those associated with the salicylic acid (SA) or jasmonic acid (JA) pathways. The relative expression levels of genes encoding PR proteins (*PR-1, PR-3, PR-4, PR-5, PR-10*) and other genes associated with stress responses (peroxidase, superoxide dismutase, lipoxygenase and glutathione-S-transferase) were measured using quantitative real-time PCR. Four varieties of maize with different susceptibilities to *M. arenaria* infection were examined at three different time points after inoculation. We found differences in plant responses between the different maize varieties and between the different time points. Most of the genes examined were down-regulated during the earliest stage of infection, and their expression increased 3 d after inoculation. The most obvious differences in expression responses between tolerant and susceptible maize varieties were found for the *PR-3, PR-4* and *PR-5* genes. We also found significant differences in responses to *M. arenaria* infection in monocotyledonous maize in comparison with previous reports in dicotyledons. Our results suggest a role for *PR-3, PR-4* and *PR-5* in maize resistance to *M. arenaria* and the importance of both the SA- and JA-mediated pathways in maize plant defenses.

1. Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops, and is used for human and animal nutrition in many countries. Over 60 nematodes species have been found that are associated with maize in different parts of the world [1]. The main genera of plant parasitic nematodes with significant economic importance are the root-knot nematodes (RKN, *Meloidogyne* spp.), the root-lesion nematodes (*Pratylenchus* spp.) and the cyst nematodes (*Heterodera* spp.) [1]. One of the root-knot nematodes that is found on maize is *Meloidogyne arenaria* [2]. The host range of *M. arenaria* is very large and includes members from many plant families; including monocotyledons, dicotyledons, herbaceous and woody plants. It is widely distributed around the world and is found in tropical, subtropical and temperate climates [3].

RKNs are the most economically important group of plant-parasitic nematodes worldwide, and have a large host range that includes over 3000 plant species [4]. Control of these nematodes constitutes a major challenge for the agricultural industry [4,5]. Nematodes from this group are invasive as second-stage juveniles (J2). They hardly ever cause necrosis, but instead dwell in the soil and mechanically penetrate the root tip epidermis to move inside the plant through the intercellular spaces. After invading the plant, they transcriptionally reprogram parenchymal cells surrounding the phloem to establish giant cells that serve as nutrient sinks for feeding [6]. The presence RKN compromises root function [6]. Moreover, during the infection process, the nematodes induce changes in patterns of gene expression, both locally and systemically throughout the plant [7]. RKN cause strong plant responses by migrating between the root cells. Plant usually recognize and react to the parasite by switching on defense responses [8]. The expression of genes encoding plant defense proteins, generally known as pathogenesis-related (PR) proteins, and other changes during the plant defense response are mainly regulated by phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) [9]. At least 17 families of PR proteins with different functions in plants have been described [10]. SA-dependent signaling activates defense genes such as *PR-1*, *PR-2* and *PR-5*, and the JA-dependent pathway is associated with increased expression of genes encoding *PR-3, PR-4* and *PR-12* [11]. The PR-protein-mediated response to pathogen infection is usually systemic

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Table 1
Target genes analyzed in this study; including GenBank accession numbers, primer sequences and annealing temperatures.

Target gene	GenBank number	Forward primer	Reverse primer	Annealing temperature
Leunig (reference)	NM_001158123	GTCAGGAACCCCAACCCTAT	CTCCCAACACCACCTTGATT	61 °C
FPGS (reference)	NM_001350861	ATCTCGTTGGGGATGTCTTG	AGCACCGTTCAAATGTCTCC	61 °C
PR-1	DQ147146	TAGCGTGCCTCCTAGCTCTG	GAGCCCCAGAAGAGGTTCTC	60 °C
PR-3	S82314	AGCTTCCTCGTCTCATCATCG	TGTTGACGTAGGCGTAGAGC	60 °C
PR-4	NM_001157282	ACGGCAAGAACCCCAACTC	CACCGACGCGCATATCAGAA	60 °C
PR-5	EU725133	CAGCCAGGACTTCTACGACA	CCATGCATGCAGAGCCTATT	60 °C
PR-10	AY953127	CAAACAGCTAAGCGCACGAC	CGATGAGCGTGTTCTTGCAC	60 °C
Catalase	NM_001111946	CAAGCCCAACGACTTCAAGC	CCATGCCATCCACGTACCTT	59 °C
Peroxidase	EU964425	CTCATCAACCACCCGGACAC	TGCAGCAAGCCTTATTTGAACA	59 °C
Superoxide dismutase	EU959305	GTCTTCGCCGCTCCCTATTC	TGGAAGCCATGAAGTCCAGG	63 °C
Lipoxygenase	NM_001111533	GGAACTTCACAGAGCAGGCT	TACGGGTAGTCCTCCACCAG	62 °C
Glutathione-S-transferase	NM_001112123	TCGAGGAGGTGACTGGAGTG	ACGTTGCCTTGGCAAATGTC	62 °C

because PR proteins not only accumulate at sites of pathogen location, but also in non-inoculated parts of the plant [12]. Defense responses and metabolic changes in systemic tissues are very energy- and nutrient-consuming for the host plant, and can lead to reduced biomass production and crop yield losses [13].

Besides PR proteins, several other proteins that are expressed during plant responses to nematode infection have been studied. Proteins associated with responses to wounding or nematode infection include peroxidase, catalase, superoxide dismutase, glutathione-S-transferase [14], lipoxygenase [15], glucanase [16], trypsin inhibitor [17] and various others [8]. There are also some studies about genes involved in *Arachis* spp. resistance to *M. arenaria* infection [18,19]. The majority of recent studies on plant defense responses after infection with RKN are in dicotyledonous plants. Data on monocotyledonous plants have mainly been obtained in rice-nematode pathosystems [13,20–23]; therefore, further information on plant defenses in other monocotyledonous plants is required. A comparison of responses to *M. graminicola* in susceptible and resistant rice (*Oryzae sativa*) varieties undertaken by Kumari, Dutta [22] analyzed expression levels of genes associated with SA-related, JA-related, ET-related and general defense responses. However, this study focused on different genes from those selected in our study. There are also some studies on maize hybrids resistant to *M. arenaria* and *M. incognita* infection [2,24]. However, the mechanism of infection were not previously analyzed, except for involvement of one gene - *Phenylalanine Ammonia Lyase* (*PAL*) in maize – *M. arenaria* interactions [25].

The aim of this study was to analyze the expression levels of genes encoding selected maize proteins (PR-1, PR-3, PR-4, PR-5, PR-10, peroxidase, superoxide dismutase, lipoxygenase and glutathione-S-transferase) that are known to be involved in plant biotic stress responses, and are associated with SA- and JA-mediated pathways, during the early stages of *M. arenaria* infection. Differences in gene expression between maize varieties with varying susceptibility to *M. arenaria* infection were also analyzed. We found that the expression levels of some of these genes were dependent on the susceptibility of the maize variety. We also detected down-regulation of the genes encoding most of the defense-related proteins in the earliest stage of infection. This finding was in contrast to previously reported responses of dicotyledonous plants, such as peanut or *Arabidopsis thaliana* [26,27], for which no gene down-regulation was observed 1 d after infection with RKN.

2. Material and methods

2.1. Organisms

Analyses were carried out using an *M. arenaria* population obtained from the Institute of Plant Protection - National Research Institute Collection (Poland) and four varieties of maize seedlings: PR39F58 (Pioneer), PR39A98 (Pioneer), Tasty Sweet (Seminis) and Multitop (Syngenta).

2.2. Plant growth, inoculation and sample collection

Plants were grown at constant day/night temperatures of 25 °C/20 °C and controlled light conditions to maintain a constant day length. Following germination, 3–4 week old seedlings at the 4–5 leaves stage were inoculated with *M. arenaria* J2 larvae that had been extracted from *Nicotiana tabacum* roots using NaOCl [28]. Approximately 1500 larvae suspended in water were used to inoculate each plant. Healthy plants were simultaneously grown as a negative control. Root samples from healthy and infected plants were collected at three time points: 24 h post inoculation (hpi), 3 d post inoculation (dpi) and 7 dpi. To determine the susceptibility of each maize variety, plants were inoculated with 500 larvae per plant and two months later the root symptoms and number of galls in each plant were observed and counted, respectively. For each plant total number of galls were calculated for gram of roots. Standard error was also calculated.

2.3. RNA extraction and cDNA synthesis

Total genomic RNA from healthy and infected root samples was extracted using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) and the concentration of each sample was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). From each sample, 200 µg of RNA was used as template for cDNA synthesis using a Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific). The cDNA was diluted 1:1 in water and used as template in the real-time PCR assay.

2.4. Real-time PCR assay

Quantitative real-time PCR reactions were performed for the target genes that encode PR-1, PR-3, PR-4, PR-5, PR-10, peroxidase, superoxide dismutase, catalase, lipooxygenase and glutathione-S-transferase. The primers used for amplification of each gene were designed using the maize sequences deposited in the National Center for Biotechnology Information (NCBI) database (Table 1). Expression of the *LEUNIG* and *folypolyglutamate synthase (FPGS)* genes, described previously [29], were used as a reference for data normalization.

All reactions contained 1 µL of template cDNA, 0.5 µM of each primer, 5 µL of iTaq master mix (Biorad), and sterile distilled water to a final volume of 10 µL. A control containing no DNA template was included to exclude contamination of the reagents. A LightCycler 96 thermocycler (Roche) was used with the following thermal profile: 5 min at 95 °C; 40 cycles of 10 s at 95 °C, 10 s at the appropriate annealing temperature for the primers pair being used (Table 1), and 10 s at 72 °C. The melting phase began at 65 °C and ended at 95 °C, with an increase of 1 °C at each step. All reactions were performed using three biological replicates and three technical replicates. The relative gene expression levels were calculated with the GenEx 6.0 software (MultiD Analyses AB, Sweden) using the formula: Relative