Plant Gene 3 (2015) 1–10

Contents lists available at ScienceDirect

Plant Gene

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

Characterization of *Brassica juncea–Alternaria brassicicola* interaction and jasmonic acid carboxyl methyl transferase expression

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ARTICLE INFO

Article history:
Received 14 February 2015
Received in revised form 12 May 2015
Accepted 18 June 2015
Available online 24 June 2015

Keywords:
Alternaria brassicicola
Brassica juncea
Jasmonic acid carboxyl methyl transferase
Methyl jasmonate
Necrotrophic fungus
Jasmonic acid

ABSTRACT

The mode of disease progression during *Alternaria brassicicola* infection, a prototype of necrotrophic fungal infection, was studied in mustard, *Brassica juncea*. During the disease advancement in mustard after infection with *A. brassicicola*, production of reactive oxygen species occured within 16 to 24 h in *B. juncea* cells. Severe necrosis was observed after 2 days of infection in *B. juncea* leaves with the development of necrotic DNA. The transcriptional activation of jasmonic acid carboxyl methyl transferase (*JMT*) was observed after 2 days post infection with *A. brassicicola*. Differential expression of *JMT* was observed in various tissues of mustard with the detection of the protein in mustard leaves only after treatment with jasmonic acid, but always found in young buds and to a lesser extent in opened flowers. Since *JMT* is involved in positive feedback regulation of jasmonate pathway and the jasmonic acid responsive genes, it could be a good target for achieving resistance against necrotrophic pathogens.

1. Introduction

Plants encounter several different types of pathogens. Plant immune system responds to the attack from these pathogens according to their type by activating three major pathways, which are mediated by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Attack by biotrophic pathogens triggers the accumulation of the signal molecule, salicylic acid (SA) that upregulates a number of genes, such as *PR1* (*pathogenesis-related 1*), *BG2* (*beta-glucanase 2*), and *PR5* (*pathogenesis-related 5*) (Ward et al., 1991). Hence, SA-dependent defense responses are effective in defense against pathogens such as *Erysiphe orontii*, *Peronospora parasitica* and *Pseudomonas syringae* (Cao et al., 1994; Delaney et al., 1994; Cao et al., 1997; Reuber et al., 1998). Pathogen attack could also trigger accumulation of jasmonic acid (JA) and ethylene (ET), but they activate a different set of defense effector genes, including plant defensin (*PDF1.2*) (Penninckx et al., 1998), and chitinase (Thomma et al., 1998). Generally, it has been observed that SA-dependent defense responses are effective against biotrophic pathogens (Cao et al., 1994; Delaney et al., 1994; Cao et al., 1997; Reuber et al., 1998), whereas JA-dependent defense responses are against necrotrophic pathogens (Staswick et al., 1998; Thomma et al., 1998; Norman-Setterblad et al., 2000).

Alternaria brassicicola is a necrotrophic fungus that causes a foliar disease, dark leaf spot (also called as black spot disease) on many economically important crucifer species. In addition to this, *A. brassicicola* is an excellent model system to study pathogenesis of fungal necrotrophs and therefore, can be utilized in developing strategies for effective resistance in crop plants against necrotrophic pathogens. For example, the salicylic acid hydrolase (*NahG*) expressing transgenic plants, where SA accumulation was artificially prevented, could very well retain their resistance against *A. brassicicola*, thus suggesting that SA is not necessary for resistance to this class of fungal pathogens (Thomma et al., 1998). In fact, *A. brassicicola* seems to be a good model of pathogenic necrotroph when studying the JA mediated defense signaling pathways in plants. An interesting study on how *A. brassicicola* grows on various leaf surfaces reported that its hyphae penetrated through the epidermis and the stomata in cauliflower, while the fungal growth in mustard was mostly on the leaf surface over the epidermis (Sharma et al., 2014). Similarly, another comparative analysis of infection on the susceptible host (mustard, *Brassica juncea*) and a resistant host (white mustard, *Sinapis alba*), showed that the infection on mustard lead to the accumulation of SA and enhanced expression of the SA marker gene, *PR1*, whereas the resistant host responded by upregulating JA related gene expression, accumulation of Abscisic acid (ABA) and enhanced expression of the genes involved in ABA biosynthesis. Also, the exogenous application of ABA on mustard leaves resulted in delayed progression of the disease (Mazumder et al., 2013).

Otani et al. (1998) observed that the spore suspension of *A. brassicicola* was pathogenic to *Brassica* species when germinated on the surface of *Brassica* leaves. In a detached leaf assay, the spore

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http://dx.doi.org/10.1016/j.plgene.2015.06.001

germination fluid induced water-soaking symptoms followed by brown necrotic lesions on mustard leaves susceptible to the pathogen, but did not cause visible symptoms on non-host leaves. *A. brassicicola* produced a 35 kDa host-specific toxic protein, AB-toxin, the first reported among the many host-specific toxins produced by *Alternaria* species. The spores began to germinate within 12 h and more than 80% of spores had germinated by 18 h after inoculation. The toxicity was first detected after 12 h of incubation and activity increased gradually with time until 24 h (Otani et al., 1998).

In the present study, we sought to understand two different aspects of "plant pathogen infection" triggered by *A. brassicicola*—(a) physiological changes at the site of infection on *B. juncea* leaves following inoculation with *A. brassicicola* and (b) immediate responses triggered in the host. Notably, we have observed the upregulation of *jasmonic acid carboxyl methyl transferase* (*JMT*) gene in *B. juncea* upon infection with *A. brassicicola*. JMT is a key enzyme that catalyzes the conversion of jasmonic acid to methyl jasmonate (MeJA) (Seo et al., 2001). MeJA is a semi-volatile organic compound that is involved in plant defense, inter-plant transfer of resistance and many other developmental pathways (Creelman and Mullet, 1997; Wasternack and Hause, 2002; Cheong and Choi, 2003; Wasternack, 2014). Our observations are reported in this communication.

2. Materials and methods

2.1. Preparation of spores and plant inoculation

Isolates of *A. brassicicola* were inoculated on potato dextrose agar media in Petri dishes (90 mm diameter) and incubated in the dark at 25 °C for 2 weeks. These Petri dishes were flooded with 10 ml distilled water, and the spores were collected by brushing the surface with a soft paint brush. All spore suspensions were filtered through six layers of cheesecloth to remove mycelial debris and washed three times with distilled water by centrifugation at 600 × *g* for 5 min. The spore concentration in the suspensions was adjusted to 1×10^5 spores/ml with distilled water (Bowling et al., 1994). Inoculation of the leaves of *B. juncea* plants with *A. brassicicola* was performed on 4-week-old, soil-grown plants by placing three 5 μl drops of a suspension of 5×10^5 conidial spores per ml of water on each leaf.

2.2. Detection of reactive oxygen species (ROS) by measuring H_2O_2

Leaf epidermal peels were pre-treated with 10 mM K^+-MES (pH 6.1) and 50 mM KCl solution for 30 min for stabilization under cool fluorescent light. The peels were then incubated in the same buffer containing 50 μM H_2DCF-DA (fluorescent dye, 2′,7′ dichlorofluorescein diacetate) for 10 min in PBS, pH 7.5 as described in Bass et al. (1983) at 25 ± 1 °C. To detect the formation of ROS, the strips were rinsed with incubation buffer to wash off excessive fluorophore and the guard cells were observed at 485 nm/525 nm excitation/emission wavelengths under an inverted fluorescence microscope (Optiphot-2, Nikon, Japan) fitted with a monochrome high-resolution digital cooled charge-coupled device camera. The captured images were analyzed by using NIH Image J software for Windows (Suhita et al., 2004). Fluorescence intensity was measured in pixels in a scale of 0 (darkest) to 250 (brightest).

2.3. Cloning of JMT cDNA from B. juncea

Total RNA was isolated from frozen leaves using TRI reagent (Sigma-Aldrich) as per manufacturer's instructions. Rapid amplification of cDNA ends (RACE) at the 5′ region was carried out using RACE-RT-PCR with 5 μg of total RNA from young floral buds and *Alternaria* infected leaf tissues of mustard, *B. juncea* (L) Czern & Coss cv. Pusa Jai Kisan and anchored oligo dT primer, using GeneRacer™ kit and Superscript™ reverse transcriptase (Invitrogen Corporation, USA) following manufacturer's instructions. In both the cases, ~1.2 kb *JMT* cDNA was amplified using *Brassica rapa NTR1* (floral nectar specific gene) (Accession no. AF179222) specific primers, JMT F and JMT R (JMT F: 5′ACCT CGAGATGGAAGTAATGCGAATTC 3′ and JMT R: 5′ AACCCATGGCTAAC CCATTCTTAGGAG 3′) and cloned in pTZ57R vector (MBI Fermentas, Germany). Further 5′ and 3′ RACE were performed to determine the sequence of the untranslated regions (UTRs). The sequence was submitted to the NCBI GenBank under the accession number AY667499.

2.4. Southern and Northern analyses

Total genomic DNA was isolated from young leaves of different *Brassica* species (Doyle and Doyle, 1990). Ten micrograms of total genomic DNA was digested with *Pst*I and *Xba*I and the restriction fragments were electrophoresed on 0.8% agarose gel. For Southern analysis, DNA fragments were transferred to nylon-N + membrane (Amersham Biosciences, USA) and subjected to hybridization with α^{32}P-ATP labeled partial JMT cDNA probe in Church–Gilbert buffer at 65 °C for 16 h. Stringency washes were carried out two times with 2 × SSC, 0.1% SDS at 65 °C for 5 min each, followed by three washes with 1 × SSC, 0.1% SDS at room temperature for 10 min each, and subjected to autoradiography (Meur et al., 2006). For Northern analysis, 15 μg of total RNA was denatured in 95% deionized formamide, 0.025% SDS, 0.5 mM EDTA at 55 °C for 15 min, subjected to electrophoresis on 1.2% formaldehyde-agarose gel with 1 × MOPS buffer and was hybridized in Church–Gilbert buffer (pH 7.0) at 60 °C and processed as described above (Meur et al., 2006). The gene probes used in Northern analyses were JMT and 18S rRNA.

2.5. Prokaryotic expression, purification and raising antibody of JMT

B. juncea JMT cDNA was cloned at *Xho*I and *Nco*I restriction sites of pRSET B vector and was transformed into *Escherichia coli* strain BL21 (DE3) pLysS. Finally, the transformed culture was grown at 37 °C until OD_{600} = 0.5, followed by induction with 1 mM IPTG for 3–4 h at 30 °C. The 47 kDa Histidine (His)-tagged JMT fusion protein was purified using Ni-NTA affinity chromatography. Anti-JMT polyclonal antibody was raised in rabbit with purified JMT protein.

2.6. Western blot analysis

Total plant protein was extracted by grinding 1 g of JA-treated and untreated leaves and flower buds in 3 ml of ice-cold protein extraction buffer (50 mM Tris–HCl — pH 7.5, 150 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 10% Glycerol, and protease inhibitors: 50 mg/ml TPCK, 50 g/ml TLCK, and 10 mM PMSF) and analyzed by SDS-PAGE. Protein concentration was quantified using Bradford method using BSA as standard. After electrophoresis, the proteins were transferred to a PVDF membrane (Pall Gellmann Corporation USA). Western blot analysis was carried out using anti-JMT rabbit polyclonal anti-sera. Polyclonal antibody of JMT diluted in the ratio of 1: 5000 was applied followed by horse radish peroxidase (HRP) conjugated goat anti rabbit IgG antibody (Bangalore GENEi, India). The visualization of the specific cross-reactivity was carried out with the HRP substrate TMB (3, 3′, 5, 5′-tetramethylbenzidine)/H_2O_2 for color detection.

3. Results

3.1. Disease progression during A. brassicicola infection on mustard leaves was accompanied by severe tissue damage and production of ROS

Upon inoculation of the mustard leaves with a spore suspension of *A. brassicicola* (1×10^5 spores/ml) and incubation at appropriate conditions (25 °C; 100% RH), the disease progression was observed as demonstrated in Fig. 1. One of the early symptoms of *A. brassicicola* infection was the appearance of water soaked necrotic lesions within 48 h