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Contribution of jasmonic acid to resistance against *Phytophthora* blight in *Capsicum annuum* cv. SCM334

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Abstract

Defense responses were investigated in the leaves of a cultivar of pepper, *Capsicum annuum* (cv. SCM334) resistant to *Phytophthora* blight. Jasmonic acid (JA) increased in the resistant cultivar immediately after inoculation with the pathogen, *Phytophthora capsici*, but as the levels of JA later decreased, levels of salicylic acid (SA) increased and were subsequently accompanied by hypersensitive response (HR)-mediated cell death in SCM334. Simultaneously, expression patterns of JA- and HR-related genes were analyzed. The mRNA of *catalase* and *peroxidase* (suppressing HR generation) disappeared, while the mRNA of *OPR3* (encoding JA synthesis reductase) was detected in SCM334 specifically. JA-mediated defense appears to be crucial in the resistance of pepper plants against *P. capsici*.

Keywords: *Capsicum annuum* cv. SCM334; *Phytophthora capsici*; Disease resistance; Hypersensitive response; Jasmonic acid; Salicylic acid

1. Introduction

Phytophthora blight, caused by *Phytophthora capsici* Leonian, is one of the most important soil-borne fungal diseases of pepper plants and is widely distributed throughout the world [19,33,35]. A Mexican accession, *Capsicum annuum* L. cv. 'Serrano Criollo de Morelos 334' (SCM), displays a high level of resistance against *P. capsici* [30]. Compared to sweet pepper plants, SCM has narrow leaves, thick hairs on the whole plant and small pungent fruits. Recently, six major chromosomal regions were identified as being involved in the quantitative trait of resistance [24,40]. At least one of the resistance factors, located on chromosome 5, is common to resistant pepper plants and is thought to transmit 'general' resistance against the oomycetes [41]. The resistance of Korean cultivars ('Kumkangkimjang', 'Kingkun', 'Damoakun', 'Taeyangkun', 'Hongsanho', 'Jinpum' and 'Champion') was influenced by inoculum concentration, growth stage of the plants and temperature [2,18]. On the other hand, the resistance of SCM was independent of these factors [30]. Another resistant cultivar, 'Smith-5', exhibited a hypersensitive response (HR) and accumulation of high levels of capsidiol, a phytoalexin of Solanaceae, in response to *P. capsici* [7]. However, the resistance mechanism in most cultivars is unclear.

Salicylic acid (SA) and jasmonic acid (JA) are well-known phytohormones, regulating signal pathways of plant defense responses to stress, such as wounding, exposure to ozone and insect or microbial attack [3,6]. It has been suggested that both positive and negative interactions occur between the SA and JA signaling pathways [5,28,43]. However, these fundamental pathways are mutually antagonistic [21]. Treatment of tobacco leaves with exogenous JA inhibited expression of SA-dependent (acidic) pathogenesis-related (PR) proteins, but enhanced the expression of JA-dependent (basic) PR proteins [28]. Some JA-insensitive mutants of *Arabidopsis thaliana* (L.) Heynh. have shown enhanced SA-mediated defense responses [17,20,31].

SA content increases in plant tissues after inoculation with some pathogens, and exposure to exogenous SA enhances resistance against a wide range of pathogens [34]. SA-dependent defense responses mainly regulate the biotrophic fungal pathogens, such as powdery mildew [9]. Moreover, SA is frequently associated with HR-mediated cell death [4,12,45], and contributes to controlling the timing and extent of the local response [1]. Reactive oxygen species (ROS) play a critical role in programmed cell death of plants after various stresses, including attack by pathogens. ROS scavenging enzymes such

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as ascorbate oxidase, catalase (CAT) and peroxidase (POD) are suppressed when the level of ROS is elevated in plant cells. Suppression of ROS scavenging enzymes plays a key role in the development of efficient defense responses in plants by enhancing pathogen-induced HR-mediated cell death [27].

Induction of JA was originally studied after wounding stress [10,26]. However, JA has been shown to induce PR proteins and phytoalexins after treatment with elicitors, and it has been suggested that JA plays a role in the defense mechanism [11]. JA pathways regulate responses to necrotrophic fungal pathogens, such as *Pythium*, employing a common virulence strategy, i.e. rapidly killing the host cells to obtain nutrients [44]. JA is biosynthesized from linoleic acid, which is catalyzed to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase and allene oxide cyclase in the lypoxygenase pathway. OPDA reductase (OPR3) reduces OPDA to JA [42].

Palloix et al. [30] showed leaves of resistant and susceptible cultivars displayed distinct differences in symptoms 24–48 h after inoculation with *P. capsici.* In this study, we investigate the interaction between a resistant cultivar, SCM, and *P. capsici* and compare it to that of a susceptible cultivar. Microscopic cytological observations were carried out in combination with the quantification of SA and JA and the detection of *CAT*, *POD* and *OPR3* mRNA in the leaves following inoculation with *P. capsici.*

Although JA is generally measured using gas chromatography–mass spectrometry (GC–MS) [13], Tamogami et al. [39] measured levels of JA in rice leaves using high performance liquid chromatography-tandem mass spectrometry (LC–MS/MS). Sample preparations for LC–MS/MS are much easier than for GC–MS, so this method was performed for quantifying JA using LC–MS.

2. Materials and methods

2.1. Plant materials and inoculum

Plants of a resistant cultivar of *Capsicum annuum* L., SCM334 (SCM), and a susceptible cultivar, California Wonder (CW), were grown for 40 days in a green-house to the three to four leaf stage. *P. capsici* was grown on a V8 juice medium [20% (v/v) V8 juice (Campbell soup, USA), 3 mM $CaCO_3$ and 1.5% (w/v) agar] for 7 days at 25 °C in the dark, followed by 3 days under fluorescent light at 28 °C. The zoosporangia were scraped off the medium into distilled water and cooled for 1 h at 4 °C. Zoospores were then differentiated after 2–3 h at 25 °C and suspended in distilled water at 10^5 spore/ml for use as inoculum. Water alone was used as the mock inoculation. The inoculum was dropped onto pepper leaves for microscopic observation and sprayed for phytohormone measurement and RNA extraction.

2.2. Microscopic observation

Leaves were excised at 8, 12, 16, 24 and 48 h after inoculation. Samples were cleared by boiling in a solution of lactic acid/phenol/glycerol/water/ethanol (ratio 1:1:1:1:8 by vol.) or ethanol. The leaf discs (5 mm diameter) were stained by submerging in 0.5% (w/v) cotton blue for 7–9 h, or vertical leaves were stained in an iodate solution ($ZnCl_2$/KI/I_2/water, 50:20:0.5:100, by vol.) for a few minutes. Following clearing and staining, the leaves were observed with the aid of an optical microscope.

2.3. Measurement of SA

Leaf pieces (0.3 g) were excised at 0, 0.5, 1, 3, 6, 12, 24 and 48 h after inoculation and boiled in 3 ml of 0.25% (v/v) acetic acid for 10 min. The extracts were filtered through a 0.2-μm filter (DISMIC-13_{HP}; Advantec, Japan). SA was detected using the LC–MS system (QP8000α; Shimadzu, Japan). Ten microlitres of the filtrates were injected onto an ODS column of diameter 4.6 mm and length 150 mm (Shodex C18-5A, Shoko, Japan), and equilibrated with 90% (v/v) acetonitrile containing 0.25% (v/v) acetic acid at a flow rate of 0.2 ml/min and a temperature of 40 °C. Mass detection was achieved by electrospray ionization in the negative ion mode at 1.5 kV with N_2 gas flow of 4.5 l/min at 230 °C. Peak SA was detected at 137 *m/z*. Aliquots of 5, 10 and 50 μg SA in 0.25% acetic acid were used as standards.

2.4. Measurement of JA

Leaf discs (5 mm diameter, 1 g) were excised at 0, 0.5, 1, 3, 6, 12 and 24 h after inoculation. Preparation of JA within the pepper leaves was according to Tamogami et al. [39], with minor modifications. JA was extracted from the leaf discs with 30 ml acetone containing 100 ng 2HJA as an internal standard left overnight at room temperature. After evaporation, the residues were dissolved in 3 ml of 75% (v/v) methanol and passed through a Sep-Pak Light C_{18} cartridge (Waters, USA). JA was detected using the LC–MS system described above. Five microlitres of the concentrated extract (containing 25 ng 2HJA) was injected onto an ODS column (same as that used for SA) and equilibrated with 80% (v/v) acetonitrile containing 0.1% (v/v) acetic acid at a flow rate of 0.5 ml/min and a temperature of 40 °C. The JA peak was detected at 209 *m/z*. Aliquots of 10, 25 and 50 ng JA, dissolved in the mobile phase, were used as standards.

2.5. RNA blot analysis

Total RNA was extracted from leaves with isogen (Nippon Gene, Japan) following the manual. One hundred milligrams of fresh leaves were ground in liquid nitrogen 0, 0.5, 1, 3, 6, 12, 18 and 24 h following inoculation and suspended with isogen at 50 °C. Chloroform was added at room temperature and centrifuged at 10k×*g* for 15 min at 4 °C. The aqueous phase was dissolved with isopropanol and centrifuged at 10k×*g* for 15 min at 4 °C. Precipitate of RNA was washed with 70% ethanol and dissolved in 50 μl water. Twenty micrograms aliquots of RNA were denatured at 65 °C for 10 min in a gel-loading buffer [10% (v/v) 10× MOPS (0.2 M MOPS, 50 mM sodium acetate, 10 mM