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Physiological and Molecular Plant Pathology 67 (2006) 149–154

PMPP

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

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Accepted 9 December 2005

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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0885-5765/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pmpp.2005.12.002

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

EDTA at pH 7.0), 6.475% (v/v) formaldehyde, 50% (v/v) denatured formamide], separated by electrophoresis on a formaldehyde gel [1% (w/v) agarose, 18% (v/v) formaldehyde, 10% (v/v) 10×MOPS], transferred to nylon membranes (Hybond N+; Roche, Germany) by capillary transfer and baked at 120 °C for 20 min. mRNAs were hybridized to probes at 45–50 °C overnight and washed twice at 60–65 °C in a solution containing 0.2×SSC (3 mM NaCl, 3 mM sodium citrate) and 0.1% (w/v) SDS for 15 min. cDNA probes of *CAT*, *POD*, *OPR3* and *histonH2B* were prepared by RT-PCR. Primer sequences were 5′-tgatgtttgtctcccgacag-3′ and 5′-tcctgtgctgacattcttgc-3′ for *CAT*, 5′-cggtgattattgcgatgagca-3′ and 5′-aagaaatcccct-gagccctc-3′ for *POD*, 5′-ccagggattttcacaaaggt-3′ and 5′-ggaaccagaatggagttgga-3′ for *OPR3*, 5′-ggcatttccagcaaatcaat-3′ and 5′-gacccaaaagatccccaaat-3′ for *histonH2B*. These primers were designed with GenBank accession nos. AF227952, AF442386, AF038384 from pepper and AJ278332 from tomato for *CAT, POD, histonH2B* and *OPR3*, respectively. One microgram of total RNA was extracted by Rneasy (Qiagen, Germany) and transferred to cDNA using RT-PCR high (Toyobo, Japan). The conditions of RT-PCR were as follows; 2 min denaturation at 94 °C, 15 cycles of 30 s at 94 °C, 30 s at temperatures decreasing 1 °C every cycle from 60 to 45 °C and 30 s at 72 °C, then 30 cycles of 30 s at 94 °C, 30 s at 45 °C and 30 s at 72 °C, followed by a final elongation for 10 min at 72 °C. The PCR products were isolated using QIA gel extract (Qiagen). The cDNA fragments were ligated into a plasmid, pBluescript II SK+, with Ligation Pack (Nippon Gene) and transformed to *Escherichia coli* DH5α for subcloning. The plasmids, including the insert cDNAs, were then isolated and the probes, labeled with digoxigenin (DIG)-dUTP by DIG DNA labeling mix (Roche), were amplified using PCR. The PCR procedures were 2 min denaturation at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, and 10 min at 72 °C for a final elongation. Hybridized blots were detected using the DIG luminescent detection kit (Roche). Visualized signals were analyzed with Densito Graph (DENSITO2, ATTO, Japan) with a relative control of *histonH2B*.

3. Results

3.1. Fungal behavior and reactions of the plant cells

Leaves of CW and SCM inoculated with zoospores of *P. capsici* were observed with the aid of an optical microscope. More than 80% of the mycelia of germinated zoospores had invaded the cuticle of CW leaves 8–12 h after inoculation (Fig. 1). Mycelial invasion was delayed in SCM leaves compared to CW; however, almost 80% of the mycelia had invaded the SCM cultivar 48 h after inoculation. Cell death was evident as brown spots on the leaves of CW 24 h after infection, with this area of necrosis spreading until the whole leaf disintegrated completely after 48 h. In contrast, leaves of SCM only showed a few brown cells after 24–48 h as a result of invasion points by the mycelia (Fig. 2). These brown cells inhibited the spread of mycelia in leaves of SCM.

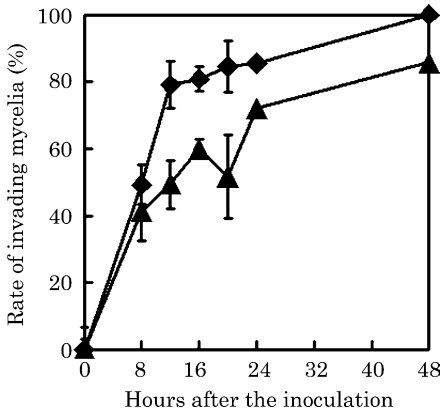

Fig. 1. Invasion rates of *P. capsici* mycelia into the pepper leaves. Rates of invading mycelia per geminated zoospore into leaves of: (▲) SCM and (♦) CW. Vertical bars represent SE.

3.2. Accumulation of SA and JA in leaves

The level of SA in the leaves was measured several times after inoculation with *P. capsici* zoospores or the mock/control (Fig. 3A). In CW leaves, there was no significant difference in the level of SA between the inoculated and mock/control with these levels remaining constant at approximately 3–4 μg/g fresh weight over time. In SCM leaves, accumulation of SA in the mock-treated leaves was low (as with those of CW), but in the leaves inoculated with *P. capsici* zoospores SA increased 6–18 h after inoculation reaching a peak of 13 μg/g at 24 h and then decreasing to levels comparative to the control at 48 h.

Levels of JA increased dramatically in the inoculated leaves of SCM (Fig. 3B). Within 0.5 h of the inoculation JA

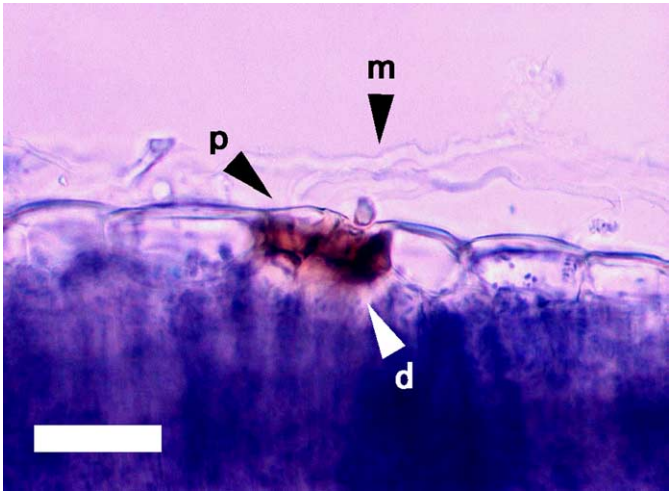

Fig. 2. An invasion point of *P. capsici* mycelium into SCM leaf tissue 48 h after inoculation and staining with iodate solution. m, Mycelia; p, invasion point; d, host cell death. Scale bar represents 100 μm.

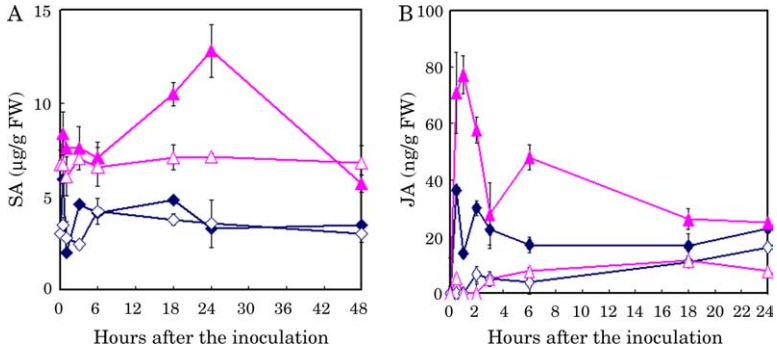

Fig. 3. Levels of (A) SA and (B) JA in pepper leaves after inoculation with *P. capsici*. Suspension of the zoospores was sprayed onto (▲) SCM and (♦) CW leaves, or mock treatment (water) onto (△) SCM and (◇) CW. SA and JA levels were measured with LC–MS. Vertical bars represent SE.

levels had increased by more than 14 times the mock-treated level. After 1 h, JA concentration reached 80 ng/g, which was the maximum value reached during the entire 24 h period. JA levels in the inoculated leaves of CW exceeded those of the mock/control, but were not as high as those observed in SCM leaves.

3.3. Expressions of CAT, POD and OPR3

Northern blot analysis revealed different expression patterns of the mRNAs between both cultivars. *CAT* expression in CW was detected within 0.5 h after inoculation and the transcript level was maintained until 3–24 h. On the other hand, *CAT* expression in SCM appeared at 1 h and gradually increased until 3–24 h following inoculation. In CW, mRNA of *POD* was detected from 0.5 h, increased until 18 h and then decreased. In SCM leaves, *POD* mRNA was not detected at any stage. Also, in SCM leaves, the *OPR3* gene was highly expressed at 0–3 h but almost disappeared 6–24 h after inoculation. In contrast, in CW leaves, *OPR3* gene expression appeared until 6–24 h after inoculation.

4. Discussion

Microscopy observations showed that invasion by *P. capsici* hyphae was more delayed in the leaves of SCM than in CW leaves (Fig. 1). This is probably due to differences in plant morphology between the two cultivars. SCM has thick hairs on the whole plant, restricting the ability of the pathogens to easily adhere to the host tissue. However, mycelia were capable of invasion later, so this effect was not sufficient for resistance to infection by *P. capsici*.

Staining of vertical leaves showed that cell death was generated in the infected cells of resistant leaves (Fig. 2). In another resistant pepper cultivar, ‘Smith-5’, HR-mediated cell death was shown to be one of the factors in resistance against *P. capsici*. The cell death in SCM leaves was very rapid and small compared to Smith-5, not to mention CW [7]. This suggests that more rapid and intense HR occurs in SCM leaves.

Levels of JA increased immediately in the pathogen-inoculated leaves of the resistant cultivar. SA content increased following a decrease in the levels of JA and associated cell death was observed. Nevertheless, there was little change in the levels of JA in the susceptible cultivar (Fig. 3). The time lag between JA and SA accumulation in SCM supports findings from previous studies, which suggest an antagonistic interaction [21,28,32,38,43]. Studies of JA signal pathways began with wounding stress [10,26]. However, Orozco-Cardenas et al. [29] showed that wounding or treatment with ethyl jasmonate induced H_2O_2, which triggers expression of plant resistant and HR-related genes. We suggest that a rapid increase in the accumulation of JA following the interaction between SCM and *P. capsici* may induce H_2O_2, which triggers subsequent HR and HR-mediated cell death.

In *Arabidopsis*, *NahG* mutants could not accumulate SA and induce *R* gene-mediated HR [4], while in soybean cells, exogenous SA accelerated the *R* gene-controlled cell death [36]. SA plays a central role in many types of cell death, including HR [45]. Therefore, the accumulation of SA, which accompanied cell death in SCM, suggests the generation of HR in the resistant cultivar.

Expression patterns of HR-related and JA synthesis genes in resistant and susceptible cultivars were analyzed. CAT and POD are reductases of H_2O_2 (the most stable in the active oxygen species), which was generated during HR [22,25]. In the susceptible cultivar, expression of the two genes was induced after inoculation and prior to symptom development or penetration by the pathogen. However, the expression was delayed or insignificant in the pathogen-inoculated SCM (Fig. 4). These ROS scavengers are possibly suppressed in the resistant plants, resulting in enhanced H_2O_2 levels followed by subsequent HR-mediated cell death. *OPR3* participates directly in the octadecanoid pathway for jasmonic acid biosynthesis, and only OPR3 can reduce the 9*S*,13*S*-stereoisomer of 12-oxophytodienoic acid, a precursor of JA [37]. *OPR3* mRNA was expressed at time zero after inoculation of SCM leaves, but the levels of JA in mock-treated SCM leaves did not increase (Fig. 3B). Expression of *OPR3* mRNA was followed by JA accumulation with a time lag, which might be due to process prior to OPR3 participation in JA synthesis.

Inoculation of the susceptible cultivar, ‘Hanbyul’, with an avirulent strain of *P. capsici* resulted in the expression of JA-inducible defense-related genes but not SA-inducible defense-related genes [15,16,23]. Other resistant cultivars, ‘Smith-5’

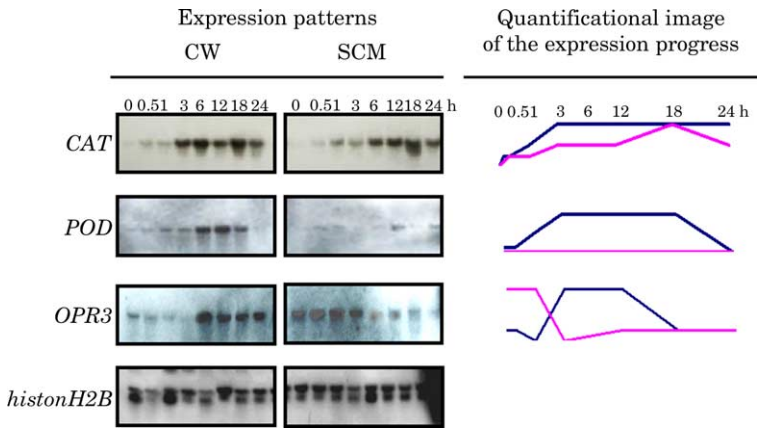

Fig. 4. Time-course of expression patterns of *CAT, POD* and *OPR3* genes in pepper leaves following inoculation with *P. capsici*. Total RNAs were extracted from SCM and CW leaves after spraying with zoospores. Aliquots of 20-μg of RNA were loaded in each lane of a 1.0% agarose gel, electrophoresed, transferred to a nylon membrane, hybridized with cDNA probes and detected as chemiluminescence. Equal loading was checked with blots hybridized with a probe for *histonH2B*. A quantificational image of the expression progress was drawn using Densito Graph (DENSITO2, ATTO, Japan) on digital graphics of the Northern blot patterns. Red and blue lines represent SCM and CW, respectively. Northern hybridization analysis was repeated at least three times.

and 'NocKwang', displayed SA-inducible defense responses against *P. capsici* for 2–9 days following infection [7,8,14]. However, there have been no studies referring to signal transduction of defense responses in strongly resistant cultivars during the early period after inoculation.

The antagonism between SA and JA is well known in plant defense responses [21,28,32,38,43], especially HR generation, which is enhanced with SA and suppressed with JA [17,20,29]. However, our results for SCM show an initial increase in JA followed by HR. Obviously, the early appearance of JA and the later accumulation of SA following inoculation of the resistant cultivar suggests that JA and SA may play a separate role in the defense response, i.e. HR-mediated cell death.

Acknowledgements

2HJA was kindly provided by Dr S. Tamogami (Akita Prefectural University). We are also indebted to Dr T. Nishijima (National Institute of Floricultural Science) and Dr M. Nagata (NIVTS) for technical advice concerning the LC–MS, to Dr H. Matsunaga (Nagano Chushin Agricultural Experiment Station) for providing pepper seeds, and to Dr K. Itoyama (Akita Agricultural Experiment Station), Dr T. Nunome and Mr H. Tsukazaki (NIVTS) for cDNA sequencing. We are grateful to Dr T. Shirakawa, Ms T. Umezawa (NIVTS), Ms Y. Mitsui (Fertilizer and Feed Inspection Service, Nagoya Office), Dr K. Abiko (JA Zennou Shiga) and Dr M. Satou (National Agriculture and Bio-orientated Research Organization, Headquarters) for laboratory support. This work was supported by 'Cooperative System for Supporting Priority Research' from the Japan Science and Technology Agency. Dr M. Hirai (Kyoto Prefectural University) and Dr S. Matsumoto (NIVTS) made great efforts to support the project. All of them except for Dr Tamogami, have assisted us through their affiliations to NIVTS.

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