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Full Paper

An investigation of a defensive chitinase against *Fusarium oxysporum* in pepper leaf tissue

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Abstract: Plant chitinase is classified as a PR-protein involved in a defense mechanism against a pathogen. This research aims to investigate a specific type of chitinase which is produced by pepper in response to an early defense against *Fusarium oxysporum*, which causes wilt disease. The changes of chitinase isozyme patterns in the inter- and intracellular fluids in the leaf of four cultivars of pepper (*Capsicum annuum* L.) at day 1, 3, 5, 7 and 10 from fungal inoculation were analysed using SDS-PAGE in polyacrylamide gel supplemented with glycol chitin as a substrate. The levels of disease severity in the four varieties of pepper were also compared with the isozyme patterns. The results showed that the resistance of pepper to *F. oxysporum* attack corresponded to the expression of ~70 kDa chitinase band (Chi-3) in the intercellular fluid. Therefore, such chitinase could possibly be used as a protein marker to identify the tolerant line and as a springboard for further study of wilt disease control.

Keywords: chitinase, isozyme, Fusarium oxysporum, PR-proteins, Capsicum annuum

Introduction

Pepper (*Capsicum annuum* L.) is an economic crop in Thailand. The major problem of pepper plantation is mostly caused by fungal pathogen. The widespread of Fusarium wilts, provoked by *Fusarium oxysporum*, has been reported to be hardly controlled. Although the host plants are removed from the farm, the fungus may be left in the soil and re-infect the plants on the next cultivation [1]. To

control such disease with a chemical pesticide can cause an environmental problem. Therefore, an investigation of a biological control method to inhibit the growth of this pathogen is a challenge.

Chitinase (EC 3.2.14) is an enzyme which catalyses the hydrolysis of chitin, the major cell wall component of many fungal pathogens [2]. Plant chitinase is characterised as one of the pathogenesis-related (PR) proteins. These proteins are induced by plants when they are infected by a pathogen. They form a protective barrier against the pathogen by collecting at infection sites and act to decrease the susceptibility of the plant [3]. Many studies have reported the success of fungal pathogen inhibition by chitinase isolated from plants [4, 5]. Transgenic plants with plant chitinase gene also show more resistance to pathogen infection [6].

Up to now, the biological control of *F. oxysporum* by chitinase isolated from pepper plant has not yet been reported. Generally, there are many types of chitinases in plants [7]. An investigation of the chitinase involved in the defense against *F. oxysporum* is therefore pertinent to the effectiveness of chitinase application. In this study, we focused on the investigation of the defensive chitinase in pepper plant by comparing the changes of the isozyme patterns with the disease severity in four cultivars of *C. annuum* L.

Materials and Methods

Chemicals and reagents

Tris(hydroxymethyl)-aminoethane-HCl (Tris-HCl) and sodium dodecyl sulfate (SDS) were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Phenylmethyl-sulfonyl fluoride (PMSF), bovine serum albumin (BSA), calcofluor white M2R, and standard proteins (high molecular weight) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Unless stated otherwise, all other reagents and chemicals were obtained from Merck (Bangkok, Thailand). All chemicals were of analytical grade.

Plants

Pepper (*Capsicum annuum* L.) cultivars (cv.) used in this study were Huay Sri Ton, T2006, Chiwalee and Hithot obtained from Known-You Seed Co. Ltd. (43 Ratchaphuek Rd., Changpuak, Muang, Chiang Mai 53000, Thailand). The seeds were disinfected with 10% sodium hypochlorite for 15 min, and followed by 30 min rinses in sterile water. Seeds were germinated in plastic pots filled with sterilised clay loam. Pots were kept in a greenhouse at 30-35°C with a 14 h photoperiod. Water was supplied daily. Sampling was done at different time intervals as required. Six-week-old pepper seedlings were used for pathogen inoculation.

Fusarium oxysporum culture

F. oxysporum used in this study was isolated from *C. annuum* by the plant pathology research group from Plant Protection Research and Development Office, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. The culture was maintained on Potato Dextrose Agar (PDA, Himedia) at 35°C. Routine subculture was achieved by transferring a mycelial plug onto a fresh PDA plate.

Plant inoculation and disease assessment

The conidial suspension of *F. oxysporum* was prepared from the 7 day-old culture by washing the surface of colonies with sterile distilled water and scraping with a scalpel, then filtering through two layers of cheesecloth to remove mycelial fragments. The number of conidia in the suspension was adjusted to 10^7/ml following counting with a haemacytometer. The plants were inoculated by stem inoculation modified by the method of Sharma [8]. The stem was vertically cut (1 to 1.5 cm long) at 2 cm above the soil line with sterile sharp scalpel and then inoculated with 1 ml of *F. oxysporum* conidia suspension. After inoculation, the wound was sealed with a wet cotton swab and then covered with a plastic strip. For control, distilled water was used instead of conidia suspension. The inoculated and healthy plants were grown in a greenhouse under the condition described above.

The disease severity of Fusarium wilts in pepper plants was rated after inoculation of the fungus based on the following scale: 0 = no disease observed, 1 = slight stunting, 2 = slight stunting and chlorosis of leaves, 3 = $\leq$10% of the leaves showing chlorosis and/or 10% of the plant with wilt symptoms, 4 = 11-25% of the plant with wilt symptoms, 5 = 26-50% of the plant with wilt symptoms, 6 = 51-100% of the plant with the wilt symptoms or plant death [9]. The disease severity data were derived from the means of 10 inoculated plants. All experiments were repeated with similar results. Data are presented from one experiment only.

Preparation of intercellular fluid (IF) and intracellular fluid (In)

The leaves of infected and control plants were harvested at day 1, 3, 5, 7 and 10 after inoculation. Individual leaves from similar position on each plant were collected and pooled at each sampling time to account for variation in chitinase levels. The isolation of intercellular fluid (IF) of pepper leaves was carried out according to De Wit and Spikman [10], with slight modification. Briefly, a gram of harvested leaves was washed with distilled water three times and then submerged in 100 ml of a mixture of ice-cold 25 mM Tris-HCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 10 mM PMSF pH 7.8 in a vacuum flask under vacuum for 5 min. The leaves were gently blotted dry using paper towels and transferred into a 3 ml plastic syringe barrel. The syringe barrel was then sat on an eppendorf tube (without a lid). After centrifugation at 4000 rpm for 15 min at 4°C, the IF was recovered in the eppendorf tube and used immediately or stored at -80°C. The entire IF-free leaves were then frozen in liquid nitrogen and ground in a mortar containing 0.1 M sodium acetate buffer pH 5.2 (2.0 ml/g fresh weight) and sand. The homogenate was centrifuged for 10 min at 3000 rpm, followed by 20 min at 10000 rpm (4°C). The supernatant (intracellular fluid, In) was collected and used immediately or stored at -80°C.

Determination of protein concentration

The protein concentration of In and IF was determined using the method described by Bradford [11] with BSA as a standard.

Separation and detection of chitinase isoenzymes by SDS-PAGE

The In and IF of infected or control leaves were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% separating gel containing 0.04% (w/v) glycol chitin under non-reducing conditions as described by Trudel and Asselin [12]. Samples (15 μg protein) were denatured by boiling for in the denaturing buffer for 5 min. After electrophoresis, gels were incubated for 20 h in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 to remove SDS from proteins and gels. Chitinase activities on gels were revealed by fluorescent staining using 0.01% (w/v) calcofluor white M2R in 50 mM Tris-HCl (pH 8.9) for 5 min, then washing with distilled water several times and destaining in distilled water for 2 h at room temperature. Gels were visualised under UV light; bands with lytic activity appeared as dark zones under UV light. To determine the apparent molecular weights of chitinase bands, the gels were further stained with Coomassie brilliant blue R-250. Bands with lytic activity appeared as white zones with blue background under daylight and were then compared with standard proteins (high molecular weight).

Results and Discussion

External symptoms of Fusarium wilt disease

To determine the resistance and susceptibility of pepper cultivars used in this study, disease severity was examined. The disease progress curves for pepper plants inoculated with conidia suspension of *F. oxysporum* are shown in Figure 1. Disease symptoms appeared 3 days after inoculation in all cultivars and directly increased with time. The development of disease symptoms in cv. Hithot was highest followed by cv. Chiwalee, T2006 and Huay Sri Ton. These results suggest that Huay Sri Ton was the best disease tolerant cultivar. In contrast, the most susceptible cultivar to pathogen attack was Hithot.

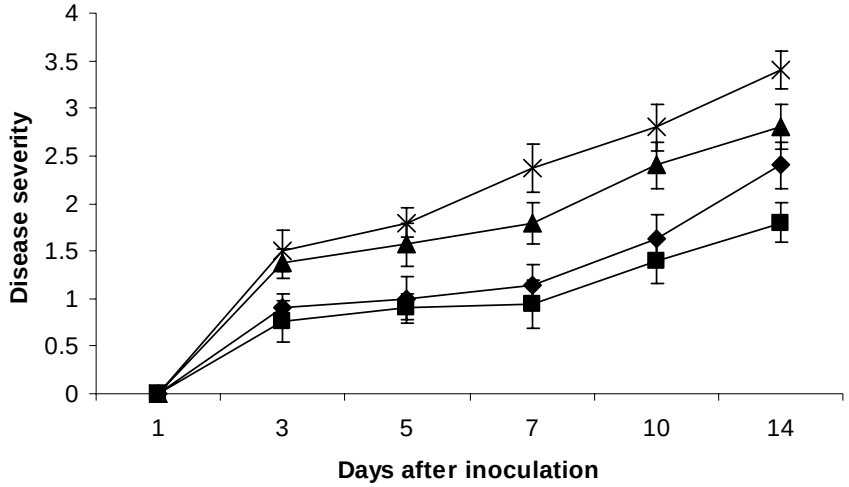

Figure 1. Disease severity of pepper plants after inoculation with *F. oxysporum*: ◆, T2006; ■, Huay Sri Ton; ▲, Chiwalee; ×, Hithot. Each bar represents a mean ± standard deviation from three replicates.

Chitinase isozyme of SDS-PAGE gels

IF and In protein extracts prepared from pepper leaves were separated on non-reducing SDS-PAGE. Glycol chitin was supplemented in the gel to detect bands of chitinase activity. Bands showing chitinolytic activity but differing in molecular weight are definded as chitinase isozyme. The chitinase isoenzyme patterns were examined at 0, 1, 3, 5, 7 and 10 days after inoculation because the early defense by chitinase in plant after pathogen inoculation has been reported [13]. In order to investigate the occurrence of chitinase in response to *F. oxysporum* in pepper plant, the changes of chitinase isozyme in inoculated plants were compared with the control. The SDS-PAGE results of each cultivar are shown in Figures 2-5.

Chitinolytic activity bands were detected at the day of inoculation in all cultivars. Although a plant may have never been infected, it nevertheless usually produces chitinase in order to protect itself from pathogen invasion [14]. The number of chitinolytic bands detected in IF and In of un-inoculated pepper plant varied. In IF, four chitinolytic bands were detected in cv.T2006 while the rest showed three chitionolytic bands. In contrast to IF, three chitinolytic bands were detected in In of cv.Huay Sri Ton, while the rest showed four chitinolytic bands.

However after inoculation with *F. oxysporum*, the change in the number of chitinolytic bands was found in cv.Huay Sri Ton. Interestingly, one band (ChiH-3) was significantly induced in both IF and In at 1 day after inoculation. Although no change of chitinolytic bands occurred in cv.T2006, Chiwalee or Hithot, the induction of Chi-3 was observed. Table 1 summarises the induction of Chi-3 detected on the gel of each cultivar. It can be seen that the disease severity was related to the induction of Chi-3 particularly in IF. This evidence was also observed in resistant cultivar of sorghum in response to fungal infection [15]. Liao *et. al.* [16] explained that the early defense against pathogen infection in plant mostly involve PR-protein found in IF.

Table 1. Induction of Chi-3 in intercellular fluid (IF) and intracellular fluid (In) of pepper cultivars after inoculation with *F. oxysporum*

Cultivar	Disease severity	Source	Induction of Chi-3	Day of appearance
Huay Sri Ton	1	IF	++	1
		In	+	1
T2006	2	IF	++	7
		In	+	7
Chiwalee	3	IF	-	-
		In	+	1
Hithot	4	IF	-	-
		In	-	-

Note: Level of induction is indicated by number of +. No induction of Chi-3 is shown by -.

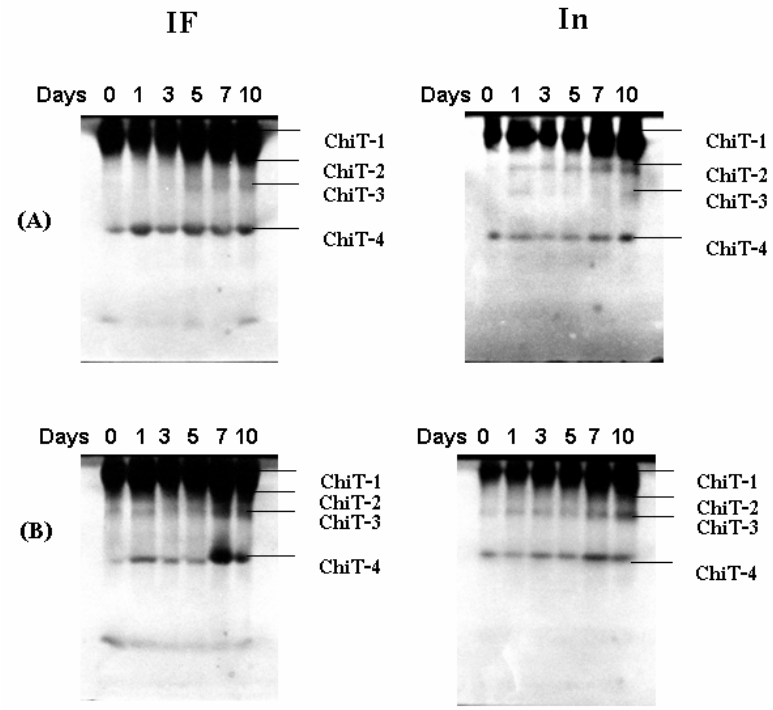

Figure 2. Chitinase isozyme patterns of intercellular fluid (IF) and intracellular fluid (In) from pepper leaves (cv.T2006) at 0, 1, 3, 5, 7 and 10 days after inoculation with *F. oxysporum* (B) comparing with water-treated control (A). All lanes were loaded with 15 μg proteins. Proteins were separated on 10% SDS-PAGE supplemented with glycol chitin. Bands with chitinase activity are indicated.

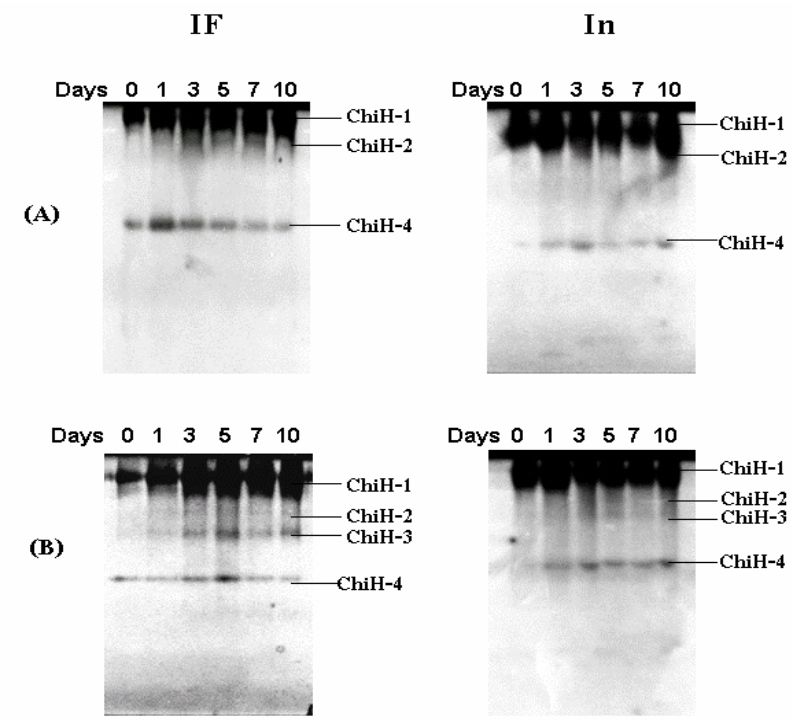

Figure 3. Chitinase isozyme patterns of intercellular fluid (IF) and intracellular fluid (In) from pepper leaves (cv. Huay Sri Ton) at 0, 1, 3, 5, 7 and 10 days after inoculation with *F. oxysporum* (B) comparing with water-treated control (A). Proteins were separated as stated above. Bands with chitinase activity are indicated.

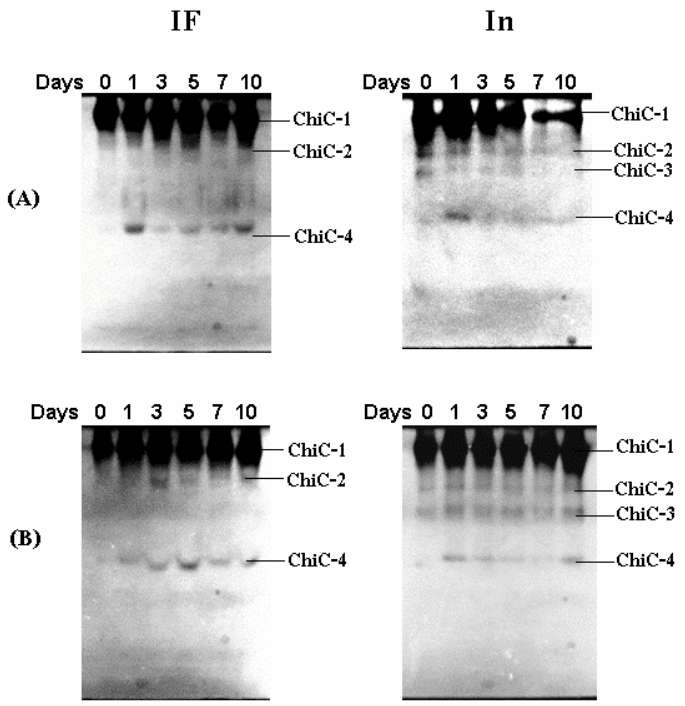

Figure 4. Chitinase isozyme patterns of intercellular fluid (IF) and intracellular fluid (In) from pepper leaves (cv. Chiwalee) at 0, 1, 3, 5, 7 and 10 days after inoculation with *F. oxysporum* (B) comparing with water-treated control (A). All lanes were loaded with 15 μg proteins. Proteins were separated on 10% SDS-PAGE supplemented with glycol chitin. Bands with chitinase activity are indicated.

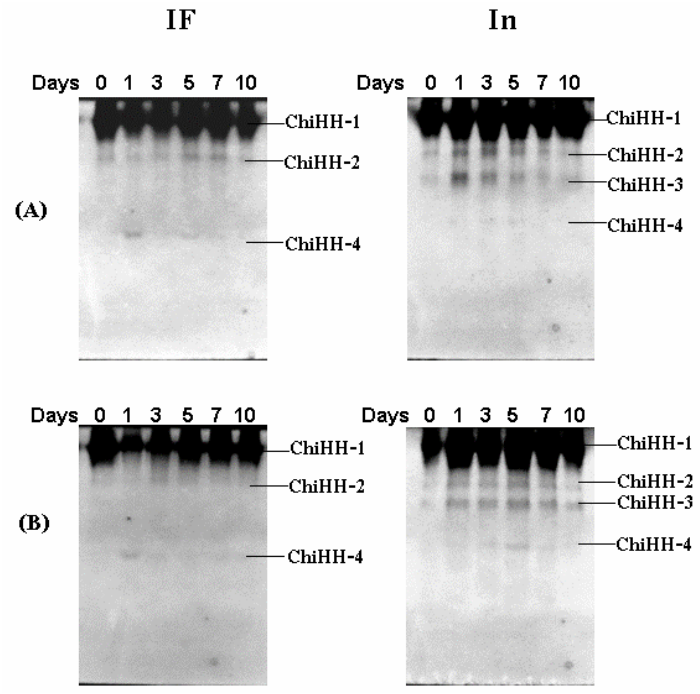

Figure 5. Chitinase isozyme patterns of intercellular fluid (IF) and intracellular fluid (In) from pepper leaves (cv. Hithot) at 0, 1, 3, 5, 7 and 10 days after inoculation with *F. oxysporum* (B) comparing with water-treated control (A). Proteins were separated as stated above. Bands with chitinase activity are indicated.

To determine the molecular weight of Chi-3, Coomassie staining was conducted. The result shows that the molecular weight of Chi-3 is approximately 70 kDa (data not shown). This protein could possibly be used as a protein marker to distinguish between resistant and susceptible cultivars of pepper plant. The chitinase band (~30 kDa) detected from chitinase isozyme was reported to successfully determine the *A. solani* resistant cultivar of tomato [17]. To use Chi-3 as a biological control agent, the study of *F. oxysporum* inhibition by this chitinase needs to be further carried out.

Conclusions

A chitinase responded in defense against *F. oxysporum* in pepper plant (*C. annuum* L.) was investigated by comparing the chitinase isoenzyme patterns in the intercellular and intracellular fluids obtained from the leaves of inoculated and un-inoculated plants. Disease symptoms in four cultivars of pepper plants were also evaluated. The result suggests that a chitinase (~70 kDa) in the intercellular fluid might be responsible for a defense against *F. oxysporum* in pepper plant.

Acknowledgements

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