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Expression of Class II Chitinase Gene in Chilli (*Capsicum annuum* L.) as Response to *Fusarium oxysporum* Pathogen Attack

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ABSTRACT

Chilli plants have many mechanism defenses to pathogen attack. The biotic stress can induce genes encoding Pathogenesis Related (PR) proteins to increase expression. The aim of this research is to analyze a molecular responds of *CaChi2* gene as PR protein in chilli, which is infected by fungal pathogenic *Fusarium oxysporum*. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure the fluorescence and the ddCt method was used to analyze the expression level of *CaChi2* in chilli. The result showed, up-regulated expression of resistant plants but no change in expression of susceptible plants after many range of time. It is concluded that the *CaChi2* gene can be a molecular marker of resistance to *Fusarium oxysporum* in chilli.

Key words: Chilli, chitinase, *Fusarium oxysporum*, qRT-PCR, resistance

INTRODUCTION

Plants have many mechanisms to defense from pathogen attack. Biochemical defense is developed by producing degradation enzymes to press or to kill their pathogen. One of the degradation enzymes is chitinase. The plant chitinase degrades β -1-4-N-acetylglucosamine that compose the fungal cell wall (Hong and Hwang, 2005; Ahmed *et al.*, 2012b). The chitinase gene can be found specifically on certain tissue or constitutively on all tissues (Kasprzewska, 2003). Chitinase is expressed constitutively at a low level but can be induced by pathogen infection (Hong and Hwang, 2005).

Chitinase is a Pathogenesis Related (PR) protein of plants. The PR protein expression can be induced by biotic or abiotic factors (Hong and Hwang, 2005; Ahmed *et al.*, 2012a). The pathogenic fungi with chitin cell wall is one of an inducer to plant chitinase expression. *Fusarium oxysporum* infection has induced many isozyme of chitinase in tomato (Chaiyawat *et al.*, 2008), whereas *Colletotrichum coccoides* has induced chitinase and β -1-3-glucanase expression in chilli (Hong and Hwang, 2005). The chitinase expression in infected *Acacia coa*, *Brassica rapa* and transgenic tobacco enhanced the plant resistance to pathogenic *Fusarium* attack (Ntui *et al.*, 2011; Ahmed *et al.*, 2012b; Rushanaedy *et al.*, 2012).

Study of molecular genetic on disease resistance is important in plant biology. Two reasons for renewing this study are the limitation of financial resources in pesticides or fungicides dependently and the considerable environmental damage. This research will study chitinase expression in chilli quantitatively based on real time PCR technique. The qRT-PCR is sensitive enough to quantify

mRNA level and widely used in plant responses to biotic or abiotic stresses (Pulla *et al.*, 2011; Orłowska *et al.*, 2012). By this technique, the target gene was specific and measured “real time” so, the result will be more accurate.

MATERIALS AND METHODS

Plant materials: Plant materials were TM999 (seeds produced by Seminis, Monsanto, Korea), Lembang-1 and Kencana (seeds produced by Crops Research Institute, Lembang, Indonesia), Cipanas, Branang and Gantari cultivar (seeds produced by Horticulture Research and Development Institute, Ngipiksari, Yogyakarta, Indonesia) that grown in sterile soil.

Fungal inoculation and disease severity index of plant: *Fusarium oxysporum* was grown in PDB for 4 days. Conidia densities were calculated using haemocytometer and adjusted to 10^6 conidia mL^{-1} . The conidia were inoculated in chilli plant by root dip method (Herman and Perl-Treves, 2007; Karimi *et al.*, 2010). One month healthy chilli plants were dug from the soil, root were rinsed in water, soaked in 1% chlorox for 1 min, rinsed with sterile water and then soaked in fungal suspension for 30 min. This treatments were done for 10 plants of each cultivar. Soaking root of health plant in sterile water was done as control. After that, each plant was planted in sterile soil in polybags. Disease symptoms were observed every odd days post inoculation (dpi) for 15 dpi. Symptoms were remarked by scoring. Score 0 = No symptom, 1 = Lower height compared to control, 2 = Lower height and chlorosis, 3 = 10% chlorosis and/or 10% wilting, 4 = 11-25% wilting, 5 = 26-50% wilting and 6 = 51-100% wilting and dead. The Disease Severity Index (DSI) was determined (Wongpia and Lomthaisong, 2010):

$$\text{DSI} = \frac{\text{Disease severity scale} \times \text{number of plants in each scale}}{\text{Highest numerical scale index} \times \text{total number of plants}}$$

The treatments repeated three replicates with ten plants of each replicate. Based on their DSI, plants were categorized as Very Resistant (VR) if $0 < \text{DSI} \leq 5\%$, Resistant (R) if $5 < \text{DSI} \leq 10\%$, Susceptible (S) if $10 < \text{DSI} \leq 30\%$ and Very Susceptible (VS) if $30 < \text{DSI} \leq 100\%$ (Suryotomo, 2006). One way ANOVA and Duncan test were used to analyze the result.

RNA isolation and one step qRT-PCR: The RNA isolation and gene expression analysis were done at 0, 4 and 15 dpi. The RNA isolation was done from 30-50 mg leaves of resistant and susceptible cultivar with total RNA mini kit plant (Geneaid) according to the protocol. The quantity and quality of the RNA were measured by nanovue spectrophotometer.

The RNA were amplified by qRT-PCR through one step qRT-PCR with KAPA SYBR Fastone step qRT-PCRkit (KAPA Biosystems), according to the protocol. The 18S rRNA gene was used as, normalized gene and the *CaChi2* gene as target gene (gene of interest = GOI). Primer pairs to qRT-PCR were 18n for normalized gene and c2p for target gene. Sequences of the primers were forward 18n 5' GGGCGACTAATGAACCCCAA 3' and reverse 18n 5' AAGCACACGTCCGCTTGATA 3' with 103 bp PCR product, forward c2p 5' CACCAGCAGATAGGTCAGCA 3' and reverse c2 p5' TCCAGTGG GAACATTCAACA 3' with 158 bp PCR product. Amplifications were done by Rotor GeneQ 5 Plex (Qiagen). One step qRT-PCR was programmed at 42°C-5 min mn for cDNA synthesis, 95°C-3 min mn for inactivate RT, with 40 cycles amplification at 95°C-3 sec for denaturation, 55°C-20 sec for annealing and 72°C-30 sec for extension.

Chitinase gene expressions of the treated plant were compared to the control plant as calibrator. Analysis of the expression based on the Ct values of GOI and normalized gene. The Ct values were analyzed by delta Ct method (Livak and Schmittgen, 2001) with rotor geneQ software version 2.1.1.

RESULTS

Pathogenic *F. oxysporum* caused wilting of the chilli plants. Among six cultivars in this research, there were not fully resistant of the plants. The symptoms were developed since 3 dpi to death at 15 dpi in susceptible plants. Based on the DSI at 15 dpi, Branang cultivar was resistant with DSI 5,7% and Lembang-1 was very susceptible with DSI 34,4%. First symptom and DSI values of six cultivars were shown in Table 1. Symptom observation on Branang and Lembang-1 were shown in Fig. 1.

There were many differences between a resistant plant and a susceptible plant in response to the *Fusarium oxysporum* pathogen attack. The first symptom in susceptible plant was seen at earlier time compared to the resistant plant. Root of the resistant plant grew densely, white and

Table 1: Disease severity index of chilli plants at 15 dpi as response to pathogenic *Fusarium oxysporum*

Cultivar	First symptom (...dpi)	DSI (%)*	Annotation**
TM999	3	22.1 ^{ab}	S
Lembang-1	1	34.4 ^a	VS
Kencana	3	27.7 ^{ab}	S
Branang	5	05.7 ^b	R
Gantari	5	15.4 ^{ab}	S
Cipanas	3	17.2 ^{ab}	S

*Number with the same letter in this column means not difference statistically with p<0.05, **S: susceptible, VS: Very susceptible, R: Resistant



Fig. 1(a-b): Symptom observation in Branang (a) Resistant plant (b) Lembang-1 (very susceptible plant)

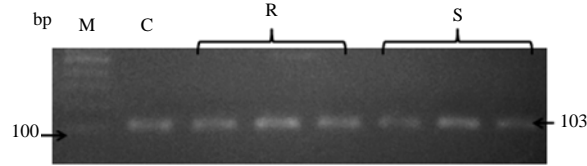


Fig. 2: Product amplification of 18S rRNA gene as normalizer. M: 100 bp DNA ladder, C: Untreated plant, R: Resistant plant, S: Susceptible plant

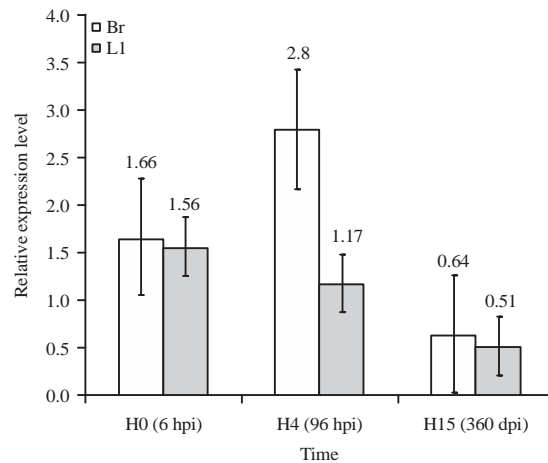


Fig. 3: Relative expression level of *CaChi2* gene in chilli plants after 0, 4 and 15 dpi of *F. oxysporum* infection. Br: Branang as resistant cultivar, L1: Lembang-1 as very susceptible cultivar. The error bars represent the standard error of the means of five replicates

healthy but thin and brown in the susceptible plant. Stem of the resistant plant was strong without necrosis but stem of the susceptible plant was weak and there was necrosis (browning) at the basal of the stem. Necrosis on the basal stem was a specific marker in *Fusarium* wilt of plants (Agrios, 2005). Leaf of the resistant plant was green and healthy but leaf of the susceptible plant was wilting.

Chitinase gene expression was measured quantitatively based on Ct values of the normalized gene and the *CaChi2* as target gene. The 18S rRNA gene was used as normalized gene in this research, because the gene was expressed constitutively in the resistant plants, susceptible plants and untreated plants (Fig. 2). The Ct values of the 18S rRNA gene were used to normalize the target gene in delta-delta Ct method. Use of the 18S rRNA gene conformed with previous research on chitinase study in *Acacia coa* infected by *F. oxysporum* (Rushanaedy *et al.*, 2012).

Based on delta-delta Ct method, there was a different profile between resistant plant and susceptible plant. Figure 3 showed the *CaChi2* expression level of the resistant and susceptible plant.

This result showed there was up-regulation on *CaChi2* gene in resistant plants but not in susceptible plants. It was confirm with previous research that expression of chitinase gene against phyto-pathogen systems is higher and induction is stronger in the resistant plants compared to susceptible plants.

DISCUSSION

Chitinase is one of the Pathogenesis Related (PR) proteins that induced by pathogen attack. Expression of the PR genes usually increases after pathogen infection (Ahmed *et al.*, 2012a). Pathogen needs time to penetrate their host plant, so the expression didn't start increasing at the earlier time. The recognition of the pathogen infection by host resistance genes should send signals to initiate various defense responses (Yu *et al.*, 2010). Study in wheat leaf concluded that there was no difference in chitinase expression of resistant and susceptible plant until 72 hpi (Mohammadi *et al.*, 2002). In that study, the symptoms could be observed visually in most susceptible plants after 3 dpi (72 hpi), when their hypersusceptible response started occur in host plants and the chitinase gene expression difference could be observed after 4 dpi.

Chitin in the fungal cell wall induced plant chitinase to be expressed highly at early infection (0-4 dpi) in resistant plant. The chitinase enzyme degraded fungal cell wall until the dead of the fungi. The chitinase expression was declined at the later (15 dpi) to basal level because of the death of the pathogen. This is confirm to the previous research in grape that the induction was steeply up to 4 dpi and declined to the basal level after 7 dpi in the resistant plant. Chitinase expression in *Sesbania rostrata* increased at 2 dpi but decreased after 7 dpi as a response to *Azorhizobium caulinodans* symbiont (Goormachtig *et al.*, 1998). According to Goormachtig *et al.* (1998), plant chitinase was produced, as a molecular response to another organism of a plant.

The susceptible plants could not up-regulated the chitinase expression so they failed to degrade fungal cell wall. The fungus penetrated in plants and colonized there but plant chitinase of susceptible plant could not degrade it. Expression level of the chitinase gene was not different compared to the untreated plant.

The expression level of chitinase gene in this research conformed to many previous researches. The chitinase was up-regulated in resistant plant of wheat, rice, grape and *Acacia coa* (Mohammadi *et al.*, 2002; Shrestha *et al.*, 2008; Vasanthaiah *et al.*, 2010; Rushanaedy *et al.*, 2012) but there was no increase in the susceptible plant. Shrestha *et al.* (2008) and Orłowska *et al.* (2012) showed that chitinase gene was induced in both resistant and susceptible plant but was induced at earlier time point in resistant compared to susceptible plant. According to that research studies, the chitinase gene is used to molecular biomarker in plant resistance against pathogenic fungi (Orłowska *et al.*, 2012; Rushanaedy *et al.*, 2012). Over expression of the gene can enhance the resistance against their pathogens. Response of chitinase gene in leaves as non-infected area can be used to quick analysis of the plant resistance without killed the plants.

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