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SHORT COMMUNICATION

Differential activation of defense-related genes in susceptible and resistant pepper cultivars infected with *Phytophthora capsici*

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Summary

This study investigated the expression pattern of genes encoding for a basic PR-1 protein, a basic β-1,3-glucanase, a peroxidase, and a sesquiterpene cyclase involved in defense responses in three pepper cultivars with different levels of resistance to *Phytophthora capsici*. All genes were up-regulated in infected stems of the pepper cultivars, with expression being detected 8 h post-inoculation. mRNA levels of these genes increased markedly by 24 h post-inoculation, and maximal induction levels were observed for the PR-1 and sesquiterpene cyclase genes. PR-1, peroxidase, and sesquiterpene genes were always expressed at higher levels in resistant cultivars than in the susceptible cultivar, although up-regulation was observed in both, suggesting that the differences between these pepper genotypes in susceptibility and resistance are a matter of the timing and magnitude of the defense response.

Introduction

Plants have evolved a number of different strategies to defend against pathogens. These strategies can be classified as either passive or active, depending on whether they are preformed, constitutive barriers, or are triggered after pathogen attack. Passive defenses include the cuticle, the cell wall, and preformed proteins and inhibitors (phytoanticipins). Once the contact has been established, active defenses are switched on, and consist of morphological barriers (cell wall thickening), secondary metabolites (phytoalexins), and defense-related proteins. Most of these inducible proteins are pathogenesis-related proteins (PRs), which have been implicated in active defense and play roles in restricting pathogen development and spread in the plant (van Loon, 1999).

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The level of resistance achieved by a plant against a given pathogen probably depends on the degree of coordination of the different defense strategies and the rapidity of the overall response. In some cases, whether a plant is susceptible or resistant can be determined by differences in the timing and magnitude of defense responses rather than by the expression of different sets of genes.

In a previous study (Silvar et al., 2005), the susceptibility of three different pepper (*Capsicum annuum* L.) genotypes to *Phytophthora capsici* (Leon.) was assessed. This fungal-like oomycete is a devastating soil-borne pathogen of worldwide distribution that infects solanaceous and cucurbitaceous hosts, causing multiple diseases (Erwin and Ribeiro, 1996). Among the pepper genotypes showing resistance to phytophthora root rot are USDA PI210234 and Serrano Criollo de Morelos 331 (SCM331), the latter being more resistant to the pathogen than the former. In contrast, Yolo Wonder is a susceptible cultivar (Gil Ortega et al., 1995; Silvar et al., 2005). A study of *P. capsici* colonization of these genotypes by real-time PCR demonstrated that USDA PI201234 and SCM331 show partial resistance (Silvar et al., 2005). However, the way these genotypes avoid extensive colonization by the pathogen is still unknown. The specific aim of the present work was to examine the expression of several defense-related genes that could potentially be related to the resistance response in these pepper cultivars.

Material and methods

Fungal and plant material

P. capsici isolate UDC196Pc was selected for this work (Silvar et al., 2006). The fungus was maintained on PDA medium for further use. Pepper cultivars Yolo Wonder, SCM331 and PI201234 were used as hosts with increasing degree of resistance to *P. capsici* (Silvar et al., 2005). Plants were grown separately in pots with 56 mL of soil in a chamber at 25 °C, with a photoperiod of 16 h light and 8 h darkness, until they were 3 weeks old.

Pathogen inoculation

A suspension of zoospores from UDC196Pc was obtained as per Silvar et al. (2005). Pepper plants were inoculated by adding 5 mL of inoculum (containing 10^4 zoospores mL^{-1}) to the soil. Samples of stems from five plants were collected at 8 and 24 h post-inoculation. Each assay was repeated twice.

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen samples using the Aurum™ Total RNA Mini Kit (BioRad) following the manufacturer's instructions. First-strand cDNA was synthesized from 100 ng of total RNA by using the iSript cDNA Synthesis Kit (BioRad).

Primer design and real-time RT-PCR assay

Primer pairs for real-time PCR were designed using the program Beacon Designed version 3.0 (BioRad), and gene sequences are available in GenBank (Table 1), except for peroxidase primers (see Fung et al., 2004) and PR1 primers (see Gayoso et al., 2007). Real-time PCR was performed in 50 μL of reaction mixture made up of 2.5 μL of cDNA, 1 × iQ SYBR Green Supermix (BioRad), and 0.3 μM of each gene-specific primer, using an iCycler iQ system (BioRad). The thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 min followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final step at 72 °C for 5 min. The actin gene was used as a constitutively expressed reference gene to normalize expression, and non-inoculated plants were chosen to represent 1 × expression of

Table 1. Real-time PCR primers used for evaluation of mRNA levels of different genes

Protein name	Accesion number	Reference	Primer		
			Name	Sequence	Amplicon
Actin	AY572427	This work	ACTFW ACTRV	5′ ATCCCTCCACCTCTTCACTCTC 3′ 5′ GCCTTAACCATTCCTGTTCCATTATC 3′	128 bp
β-1,3-Glucanase	AF227953	This work	GLUFW GLURV	5′ ACAGGCACATCTTCACTTACC 3′ 5′ CGAGCAAAGGCGAATTTATCC 3′	226 bp
Peroxidase	AF442386	Fung et al. (2004)	PXFW PXRV	5′ GGCGCCAGGATTGCTGACAA 3′ 5′ GTGGACATAATCCTCGAAGC 3′	520 bp
Sesquiterpene cyclase	AF061285	This work	SCFW SCRV	5′ GCCTCCTGCTTCTGAATACC 3′ 5′ TTAATATCCTTCCATCCCGACTC 3′	312 bp
PR-1	AF053343	Gayoso et al. (2007)	PR1FW PR1RV	5′ GTTGTGCTAGGGTTCGGTGT 3′ 5′ CAAGCAATTATTTAAACGATCCA 3′	301 bp

the gene of interest. The $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the relative expression of each gene. Each assay was repeated twice and each measurement was performed in duplicate.

Results and discussion

The expression patterns of four pepper defense-related genes encoding for a basic PR-1 protein (CABPR1), a sesquiterpene cyclase (CASC1), a basic β-1,3-glucanase (CABGLU), and a peroxidase (CAPO1) were analyzed in stems of three different pepper cultivars infected with *P. capsici*. In a previous study, we demonstrated that pathogen colonization of pepper is much heavier in stems than in other organs. *P. capsici* DNA was quantified at early time points in both resistant and susceptible cultivars, but at 24 h post-infection pathogen amount was much lower in the resistant genotypes PI201234 and SCM331 than in the susceptible Yolo Wonder (Silvar et al., 2005). The primary goal of the present study was to elucidate whether such differences in the plant response occur as a result of the activation of different defense-related genes, especially those that encode for PR proteins or play a role in phytoalexin biosynthesis.

All the defense-related genes studied were up-regulated in *P. capsici*-infected stems from all cultivars (Figure 1). The up-regulation was first observed at 8 h post-inoculation, although it was higher at 24 h, especially in the resistant and partially resistant cultivars. At this time point, a decrease (more than 20-fold) in the pathogen amount has also been reported in PI201234 and SCM331 (Silvar et al., 2005). The mRNA levels of the four defense-related genes differed, and varied markedly among the three different pepper genotypes. Within pathogenesis-related proteins, CABPR1 showed the highest up-regulation (41.7-fold in PI201234 at 24 h, Figure 1A), but the non-PR gene CASC1 was even more up-regulated (47.9-fold in SCM331 at 24 h, Figure 1B). The other PR-genes were up-regulated to a lesser extent: CABGLU 15.3-fold in SCM331 at 24 h (Figure 1C) and CAPO1 11.6-fold in SCM331 at 24 h (Figure 1D). Expression levels of CABPR1 were high in resistant and moderately resistant cultivars, especially at 24 h post-inoculation, when the amount of PR-1 mRNA was up to three times higher in SCM331 and PI201234 than in the susceptible genotype (Figure 1A). A similar trend was found for CASC1 at 24 h, when the induction of this gene was more than two and five times higher in PI201234 and SCM331, respectively (Figure 1B). The differences in CABGLU expression among the different cultivars at 8 h post-inoculation were not significant. However, at 24 h post-inoculation, this gene showed increased expression in SCM331 (12.3-fold) and Yolo Wonder (15.3-fold) compared with PI201234 (7.0-fold) (Figure 1C). Differences in CAPO1 expression among the three cultivars were observed at 8 h post-inoculation, but were much more marked at 24 h, when expression levels in PI201234 and SCM331 were two and three times higher, respectively, than in the susceptible cultivar (Figure 1D).

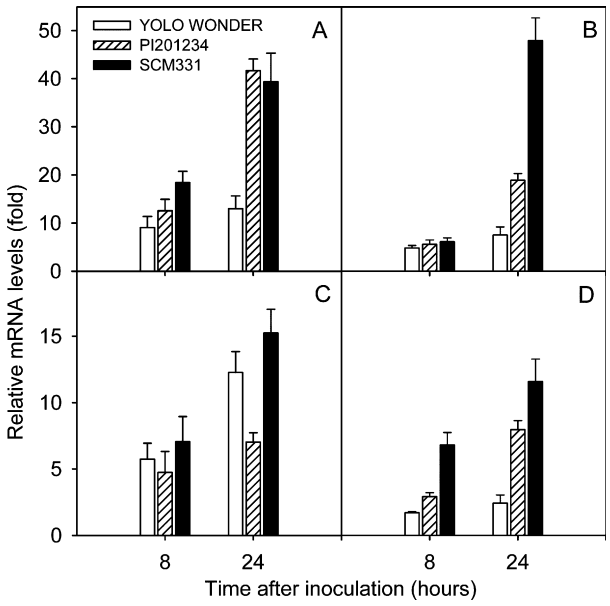

Figure 1. Relative expression levels of defense-related genes in *P. capsici*-infected stems of different pepper cultivars. (A) PR-1, (B) sesquiterpene cyclase, (C) β-1,3-glucanase, and (D) peroxidase. Data are the means and standard errors of two independent assays. Note that two different scales are used in graphs.

Inducible defense-related proteins have long been associated with plant resistance. The results obtained in the present study agree with those of Lee et al. (2000), who reported that the accumulation of CABPR1 transcripts was greater and more rapid in an incompatible interaction of pepper with *P. capsici*. Similarly, CABPR1 was strongly induced after ethephon treatment and *Xanthomonas campestris* pv. *vesicatoria* infection (Kim and Hwang, 2000), and over-expression of this gene in tobacco plants enhances tolerance to the oomycete *Phytophthora nicotianae* and the bacterial pathogens *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tabaci* (Sarowar et al., 2005). Therefore, although the precise biological role of the PR-1 proteins remains unknown, they appear to be important players in restricting pathogen colonization in resistant pepper cultivars.

With the identification of the PR-2 family as β-1,3-endoglucanases and PR-3, -4, -8 and -11 as endochitinases, the role of PR proteins in limiting

pathogen activity became clearer. β-1,3-glucanases hydrolyze the β-1,3-glucans, major components of the cell wall of oomycetes (Kim and Hwang, 1997). Glucanases may act directly by inhibiting the growth of the pathogen, or indirectly by aiding in the generation of signal molecules that may function as elicitors of further defensive mechanisms. We would expect that acidic isoforms, which accumulate predominantly in the extracellular space, play a role in the release of glucan fragments from both the pathogen and host cell walls, triggering a downstream response including cellular lysis. As a consequence, basic isoforms will be released and able to act against the pathogen (van Loon et al., 2006). This could explain why the basic CABGLU gene is particularly strongly expressed at 24 h post-inoculation. It likely acts downstream of extracellular isoforms and takes part in the suppression of pathogen development rather than in the release of elicitors for signal transduction. Jung and Hwang (2000) observed that CABGLU mRNA increases in the first stage of infection to similar levels in both compatible and incompatible interactions with *P. capsici*, but at later times, the gene was more expressed in the incompatible interaction. The marked increase in CABGLU expression seen in both Yolo Wonder and SCM331 suggests that this pepper β-1,3-glucanase may be involved in pathogenesis as well as in the disease resistance response.

Peroxidases are known to be activated in response to pathogen attacks, and various roles have been attributed to them, especially roles related to resistance (Passardi et al., 2005). On the one hand, they can create a highly toxic environment for the pathogen by massively producing reactive oxygen species (oxidative burst). On the other hand, they are involved in the deposition of cell-wall strengthening materials, such as lignin and suberin, which form a mechanical barrier against pathogenic agents. Previous work by Do et al. (2003) with the CAPO1 gene has demonstrated that this gene is more strongly induced in plants inoculated with an avirulent isolate than in those inoculated with virulent *P. capsici*. The authors suggested that expression of this gene may be related to ROS-associated defense responses, since peroxidases are closely correlated with H_2O_2 accumulation during the hypersensitive response in resistant cultivars. The rapid response observed in our experiments, especially in resistant genotypes, supports the possible role of CAPO1 in the oxidative burst that occurs in the early resistance response to pathogens. However, a role in cell-wall reinforcement through phenolic polymerization reactions cannot be ruled out. The marked increase in CAPO1 mRNA levels that we observed at 24 h in resistant genotypes could be correlated with a participation of this gene in the formation of defensive barriers, although further work would be necessary to confirm this hypothesis.

Apart from inducible proteins, active plant defenses against pathogens also include the synthesis of phytoalexins. Capsidiol is the main antimicrobial sesquiterpenoid phytoalexin produced by pepper, and is formed via the isoprenoid pathway from 5-epi-aristolochene in a reaction catalyzed by a sesquiterpene cyclase with 5-epi-aristolochene synthase activity (Whitehead et al., 1989). Our experiments showed marked up-regulation of the CASC1 gene at 24 h after infection with *P. capsici*, especially in the resistant cultivar SCM331, in which CASC1 expression level increased up to six times in comparison with the susceptible genotype. Ha et al. (2003) found that the expression of a sesquiterpene cyclase gene in pepper was strongly elevated at 24 h after infection with *P. capsici*, and was correspondingly and sequentially regulated together with a 3-hydroxy-3-methylglutaryl-CoA reductase. Both enzymes catalyze key steps in the biosynthesis defense-related sesquiterpene phytoalexins in pepper. Previous work carried out by Mandujano-Chávez et al. (2000) showed strong induction of sesquiterpene cyclase activity at 36 h after treatment of tobacco cell cultures with a fungal elicitor. This induction was clearly correlated with the capsidiol accumulation seen in the same cells. The results observed in our study support a role for this sesquiterpene cyclase gene in the resistant pepper response against pathogen attack, although more detailed work will be necessary to clarify in which pathway this gene is involved.

Together, our data strongly suggest that a correlation exists at early stages of infection between the level of resistance to *P. capsici* and the degree of the defense response at the gene expression level. The ability of virulent *P. capsici* isolates to colonize the plant and cause disease could be a consequence of a weaker expression of defense patterns in susceptible genotypes, more than an effect of qualitative differences in the reactions themselves.

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References

Do HM, Hong JK, Jung HW, Kim SH, Ham JH, Hwang BK. Mol Plant-Microbe Interact 2003;16:196–205.
Erwin DC, Ribeiro OK. Phytophthora diseases worldwide. Saint Paul, MN: APS Press; 1996.
Fung RWM, Wang CY, Smith DL, Gross KC, Tian M. Plant Sci 2004;166:711–9.
Gayoso C, Martinez de Ilarduya O, Pomar F, Merino F. Eur J Plant Pathol 2007;118:199–209.
Gil Ortega R, Español CP, Zueco JC. Plant Breed 1995; 114:74–7.
Ha SH, Kim JB, Hwang YS, Lee SW. Biochim Biophys Acta 2003;1625:253–60.
Jung HW, Hwang BK. Plant Sci 2000;159:97–106.
Kim YJ, Hwang BK. Physiol Mol Plant Pathol 1997;50: 103–15.
Kim YJ, Hwang BK. Physiol Plant 2000;108:51–60.
Lee YK, Hippe-Sanwald S, Lee SC, Hohenberg H, Hwang BK. Protoplasma 2000;211:64–75.
Livak KJ, Schmittgen TD. Methods 2001;25:402–8.
Mandujano-Chávez A, Schoenbeck MA, Ralston LF, Loyola-Gloria E, Chappell J. Arch Biochem Biophys 2000;381: 285–94.
Passardi FC, Cosio CP, Dunand C. Plant Cell Rep 2005;24:255–65.
Sarowar S, Kim YJ, Kim EN, Kim KD, Hwang BK, Islam R, Shin JS. Plant Cell Rep 2005;24:216–24.
Silvar C, Diaz J, Merino F. Phytopathology 2005;95: 1423–9.
Silvar C, Merino F, Diaz J. Plant Dis 2006;90:1135–42.
Van Loon LC. In: Datta SK, Muthukrishnan S, editors. Pathogenesis-related proteins in plants. Boca Raton, FL; 1999, p. 1–19.
Van Loon LC, Rep M, Pieterse CMJ. Annu Rev Phytopathol 2006;44:135–62.
Whitehead JM, Threlfall DR, Ewing DF. Phytochemistry 1989;28:775–9.