EXPRESSION OF A SUNFLOWER CC-NBS-LRR RGA AND DEFENSE-RELATED GENES DURING AN INCOMPATIBLE INTERACTION WITH *PLASMOPARA HALSTEDII*

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Abstract

Downy mildew, caused by *Plasmopara halstedii,* is one of the main diseases causing economic losses in cultivated sunflower. Resistance in the host is conferred by major genes denoted *Pl*. The genotype 'QIR8', which contains the *Pl8* locus, was used to isolate complete DNA genomic and cDNA RGA sequences. This RGA belongs to the CC-NBS-LRR class of plant resistantce genes. The level of transcript accumulation corresponding to this RGA was monitored by RT-PCR. The results show that this RGA is induced about three days after infection and the accumulation decreases after nine days post-infection. The high level of transcriptional expression of this RGA corresponds to the activation of a hypersensitive-like reaction. We examined the transcriptional changes of some defense-related genes that occur during compatible and incompatible combinations. In general, transcriptional activation of defense-related genes was observed in both cases. However, the induction was faster and greater in an incompatible reaction than during susceptibility. The NDR1 and EDS1, two components of R genes mediated signalling pathways as well as NPR1, an essential regulatory component of systemic acquired resistance were also tested.

Introduction

The success of pathogen infection of a host plant depends on how fast the plant recognizes the pathogen and activates the appropriate defense reaction. If the pathogen carries an avr (avirulence) gene whose product is specifically recognized by the product of the corresponding R (resistance) gene in the plant, resistance mechanisms are triggered rapidly, resulting in disease resistance. If either the avr or the R gene is absent, the pathogen is not recognized rapidly, defense responses are activated slowly, and disease ensues (Dangl and Jones, 2001). During incompatible plant-pathogen interactions, recognition of a potential pathogen often results in a hypersensitive reaction (HR), a localized activation of programmed cell death (PCD). It has recently been reported that the simultaneous production of H2O2 and nitric acid (NO) is required to induce the PCD occurring during the HR (Concetta de Pinto et al., 2002). Many physiological and molecular changes are associated with the HR. Alteration in respiration rates, electrolyte leakage and oxidative cross linking of cell wall proteins have been reported as some of the rapid physiological changes occurring during the HR. A number of defense genes whose products may intervene in this plant response have been extensively studied: they include enzymes of the phenolpropanoid pathway involved in the synthesis of

antimicrobial phytoalexins, enzymes with hydrolytic activities, toxic compounds and cell wall proteins (Lamb et al., 1989). In infected plants, these genes are induced around the necrosis once it has developed, i.e., late in HR. Moreover, most of them are also strongly expressed during compatible interactions, which result in the disease of the plant, and for some of them, during the normal development of the plant (Lamb et al. 1989). The lack of specificity of these defense genes as well as their activation in the late stages of the HR suggest that they may not, by themselves, account for the establishment of the complex inducible response that constitutes the HR, but rather may accompany this reaction.

Most plant *R* genes that have been isolated to date encode proteins that share structural similarities in that they have a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR). The NBS region is preceded by either a coiled-coil (CC) domain or a so-called TIR domain that is defined by its homology to the intracellular effector domains of the *Drosophila* Toll and human interleukin-1 receptors.

Downy mildew, caused by *Plasmopara halstedii* (Farl.) Berl. and de Toni, is one of the main diseases causing economic losses in cultivated sunflower (*Helianthus annuus* L.). The major dominant genes denoted by *Pl* confer resistance to this disease. At this time only two loci have been localized on the RFLP map of Gentzbittel et al. (1999); the first locus (Pl1/Pl2/Pl6 locus) has been localized on linkage group 1 (Mouzeyar et al., 1995; Vear et al., 1997; Roecel-Drevet et al., 1996) where as the second locus (Pl5/Pl8) has been localized on linkage group 6 (Bert et al. 2001; Radwan et al. 2003). The study of resistance to sunflower downy mildew, *Plasmopara halstedii*, revealed that there were two types of resistance (Mouzeyar et al., 1994). With type I resistance, the pathogenic agent is limited to the roots and the lower part of the hypocotyl, whereas with the type II resistance, the pathogenic agent grows throughout the whole length of the hypocotyl and may sporulate on the cotyledons. Radwan et al. (2004) used YSQ and QIR8 lines (which showed type II resistance) to clone, sequence and map two full-length sequences belonging to the CC-NBS-LRR class of plant resistance genes in sunflower.

The aim of this study was to compare the transcriptional expression of this RGA and different defense-related genes during the compatible and incompatible reactions with *Plasmopara halstedii* race 300.

Materials and Methods

Sunflower Genotypes and Infection Procedure. Infection methods were developed on the sunflower lines QIR8 (resistant) and CAY (susceptible). The infection procedure of *Plasmopara halstedii* (race 300) was carried out as described by Mouzeyar et al. (1994). The infected plants were placed, for 2 weeks, in a growth chamber at 18±1C, with a relative humidity of 60 to 70%, a photoperiod of 16h and a light intensity of about 200 µE/m sq/s. The light was provided by 250 watt Osram mercury vapor lamps.

DNA and RNA Manipulations. DNA was isolated using the CTAB method, as described by Saghai Maroof et al. (1984), whereas the RNA was extracted by using the method described by Bogorad et al. (1983) with slight modification, using only 0.5 g of the sample.

RT-PCR Procedures. Total RNA was treated twice with DNAase 1 in the presence of Rnasine (Invitrogen, France) to remove the genomic DNA contamination. Two µg of DNAase treated RNA was primed with an oligo (dT)12-18 and the first cDNA strand was synthesized using 50 units of reverse transcriptase (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, France), 2 µl of 10X RT-buffer, 4 µl of Mgcl2 (25 mM), 1

µl dNTP mix (10 mM), 2 µl DTT (0.1 M) and 1 µl of Rnasine, in 20 µl final volume. The mixture was incubated at 42C for 75 min and then the reaction was terminated at 70C for 15 min. Finally 1 µl of Rnase H was added to each tube and incubated for 20 min at 37C. A "minus" reverse transcriptase RT-PCR reaction was used as a control to test each mRNA sample for genomic DNA contamination. The cDNA synthesis and RT-PCR conditions were as described above except no reverse transcriptase enzyme was added to the cDNA synthesis reaction.

Amplification, Cloning and Sequencing of Defense Genes. Multiple sequence alignment (CLUSTAL-X, Thompson et al., 1997) of defense genes of other species listed in GenBank databases at the National Center for Biological Information (www.ncbi.nlm.nih.gov) was performed. Primers were designed within regions highly conserved to produce RT-PCR products. This method (degenerate primers) was used for all the defense genes used in this work except the PR5 and PDF1.2 (Xu et al., 2003), elongation factor of *Plasmopara halstedii* (M.F. Bouzidi, 2004, pers. comm.) and CC-NBS-LRR (Radwan et al., 2004), for which specific primers were used. The amplified products were cloned into PGEM-T Easy Vector (Promega) and sequenced by Genome Express (Grenoble, France).

RGAs and Defense Genes Expression. Upon sequence verification, specific primers were designed. Transcriptional expression of each gene was analyzed by semiquantitative PCR.

Results and Discussion

The predicated protein structure of the RGA clone Ha-NTIR11g was 1279 amino acids long and showed similarity to other resistance genes. It shared 53% identity with a resistance protein candidate (RGC 1b) from *Lactuca sativa* L. (Shen et al., 1998), 32% identity with an *RPP13*-like protein encoded by the *Arabidopsis* downy mildew resistance gene from *Arabidopsis thaliana* L. (Sato et al., 2000), 30% and 32% identity with an *I2C-2* protein encoded by the tomato wilt resistance gene from *Lycopersicon esculentum* Mill. (Ori et al., 1997). It has 9% identity with a PU3 protein encoded by a sunflower downy mildew resistance gene candidate from *Helianthus annuus* (Bouzidi et al., 2002). The amino acid sequence predicted from Ha-NTIR11g has been divided into three domains (Figure 1). The first is the CC domain (amino acids 1-154). This domain contains a leucine zipper (LZ) (amino acids 26-57). The second domain is NBS (amino acids 155-542). This domain includes P-loop (Kinase 1-a), Kinase 2 and Kinase 3-a (RNBS-B) motifs of the NBS and the RNBS-A, RNBS-C, GLPL, RNBS-D and MHD motifs that are conserved in the NBS domain. The third domain is the LRR and C terminus (amino acids 542-1279). Several LRR motifs were detected in this region; however, most of them are imperfect except for three motifs.

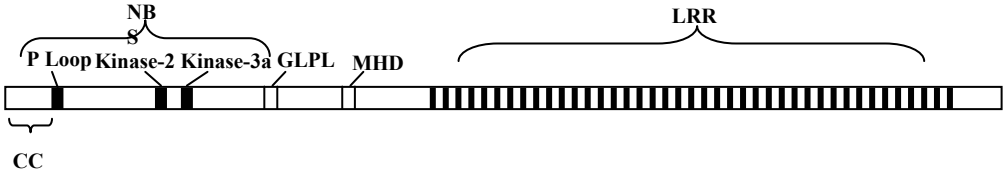

Figure 1. Diagram characterization of Ha-NTIR11g cDNA. The sequence is divided into three domains: CC 'coiled coils' domain, NBS 'nucleotide binding site' domain and LRR 'lucine rich repeat' domain. The different motifs were identified according to Moyers et al 1999.

Southern hybridization analysis was carried out to detect the genomic organization of Ha-NTIR11g. Southern blots showed multiple bands of varying intensity in each enzyme restriction digestion and these bands represented Ha-NTIR11g or its homologous sequences in sunflower. Several polymorphic bands were detected between the susceptible and resistant parents CAY and QIR8 for Ha-NTIR11g.

The level of transcript accumulation corresponding to this RGA was monitored by semiquantitative PCR. The results show that this RGA is induced about 3 days after infection and the accumulation decreases after 9 days post-infection. The high level of transcriptional expression of this RGA corresponds to the activation of a hypersensitive-like reaction. We examined the transcriptional changes of some defense-related genes that occur during compatible and incompatible combinations. In general, transcriptional activation of defense-related genes was observed in both cases (Figure 2).

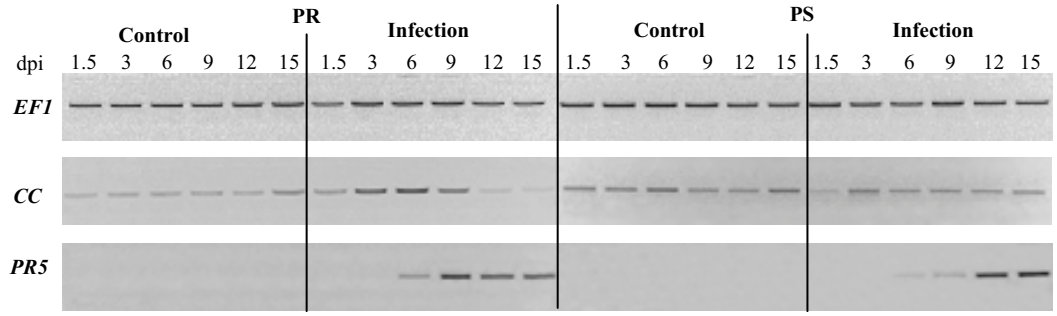

Figure 2. Semi-quantitative reverse transcription PCR. Derived amplification products were separated on TAE agarose gels and stained with ethidium bromide. EF1= constitutive elongation factor, CC= Ha-NTIR11g RGA 'CC-NBS-LRR', Control= plant uninfected, Infection= plant infected with *Plasmopara halstedii* race 300, PR= resistant sunflower line (QIR8) and PS= susceptible sunflower line (CAY)

Rapid production of reactive oxygen species (ROS), some of which may be generated by a multi-subunit NADPH oxidase complex in the plasma membrane (Doke et al., 1994), is often associated with cell death. Rapid ion fluxes across the plasma membrane, called XR (Atkinson et al., 1985), are also considered one of the earliest events of the HR. A lipid-based second messenger might also be involved in the HR-signaling process. However, is still not clear whether these signals are necessary and/or sufficient to initiate cell death. In contrast to other defense genes, HSR203j, a tobacco gene whose activation is rapid, highly localized and specific for incompatible plant-pathogen interaction (Pontier et al., 1994), has been shown to be strongly correlated with programmed cell death occurring in response not only to diverse pathogens, but also to various cell-death-triggering extracellular agents (Pontier et al., 1998). We used degenerate primers to amplify the corresponding gene in sunflower and found that this gene is induced about 3 days after infection, and the accumulation decreases after 9 days post-infection in the case of an incompatible reaction. In contrast, no activation was observed during the compatible reaction. The high level of transcriptional activation of HSR203j coincides with the activation of a hypersensitive-like reaction as well as a high level of transcriptional expression of the RGA.

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