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Cloning and characterization of peroxidases associated with generalized defense reactions of plants against bacterial pathogens

Zoltán Bozsó*, Eszter Besenyei, Péter G. Ott, Arnold Czelleng, Zoltán Klement

Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary

ABSTRACT After bacterial infection pathogens can induce different types of plant defense responses. Recently we have investigated an early type of local induced resistance (EIR). EIR is induced by both pathogenic and non-pathogenic bacteria, and is not accompanied by plant cell death (HR), but can inhibit bacterial pathogens. We have found that plant peroxidase isoenzymes were activated some hours after bacterial inoculation in tobacco. A peroxidase gene, which activated during EIR was cloned. It was proved to be a peroxidase tpoxN1, which had previously cloned from tobacco mosaic virus (TMV) infected tobacco leaves. The transcription of tpoxN1 gene was induced both by incompatible and saprophytic and was blocked by compatible *Pseudomonas* spp. and was slightly induced by water infiltration. The appearance of peroxidase proteins and its transcription activation may be a useful marker for detailed study of EIR.
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Bacterial pathogens have different elicitors which can induce various defense responses in plants after infections. In incompatible host-pathogen combinations bacteria inject Avr protein(s) into plant cells (Alfano et al. 1997). These Avr proteins interact directly or indirectly with plant resistance gene products and trigger signal cascades that activate different plant defense reactions, *e.g.* accumulation of active oxygen species, phytoalexins and finally lead to the death of plant cells (hypersensitive reaction, HR) (Hammond-Kosack and Jones 1996). Other types of elicitors are not specific for pathogen bacteria but are common in pathogenic and non-pathogenic bacteria. So far, two types of bacterial surface appendixes have been isolated from plant pathogenic bacteria which were able to trigger plant defense responses: lipopolysaccharides and bacterial flagellin proteins (Newman et al. 1995; Felix et al. 1999). These elicitors and non-pathogenic or HR-negative mutants of pathogenic bacteria also induce defense responses such as induction of defense-related gene accumulation, and plant cell wall fortification (Brown et al. 1998). This kind of defense reaction is called generalized resistance response because it is also induced by non-pathogenic bacteria and is not accompanied by HR (Jakobek and Lindgren 1993). Recently we have focused our work on the early developing form of generalized resistance response (early induced resistance, EIR), which develops approximately 6 hours after bacterial inoculation (Klement et al. 1999). The fact that pre-treatment of plant leaves with non-pathogenic bacteria or HR-negative mutants inhibits the multiplication of forthcoming injected pathogen as well as the development of disease or HR, also proved that non-HR inducing bacteria are able to induce resistance responses in plant tissues (Burgyán and Klement 1979; Klement et al. 1999).

Plant peroxidases have been extensively studied and many of their functions have been described *e.g.* scavenging of peroxide, participation in lignification, hormonal signaling and plant defense (Hiraga et al. 2001).

The plant cells express many peroxidase isoenzymes parallel. In different organs and tissues different subsets of isoenzymes are translated. For example in tobacco roots 12 peroxidase isoenzymes were detected, whereas healthy tobacco leaves expressed only 5 isoenzymes (Langrimini and Rothstein 1987).

In this study we demonstrate that at least two new peroxidase isoenzymes are activated in tobacco leaves infiltrated with HR-negative *Pseudomonas* ssp. We have cloned one peroxidase gene that is transcribed in these tissues and discuss its expression in different plant pathogen interactions.

Materials and Methods

Plant materials and bacteria

Tobacco plants (*Nicotiana tabacum* cv. Samsun nn or Xanthi NN) were grown in a greenhouse in soil. Before inoculation the tobacco plants were kept in a growth chamber for 16/8 hr light/dark periods at 20ºC for several days. After bacterial inoculation the plants were placed under continuous light.

Bacteria were grown on King's B medium at 27ºC overnight, and were suspended ($2x10^8$ cells/ml) in distilled water. The following bacteria were injected into interveinal leaf panels of tobacco with hypodermic syringes: i) *Pseudomonas syringae* pv.. *syringae* 61; ii) *Pseudomonas syringae* pv.*; syringae* 61 *hrcC* mutant; iii) *Pseudomonas tabaci*; iv) *Pseudomonas fluorescens.*

Isolation of peroxidase cDNA from tobacco

A peroxidase gene that is induced in plant tissue infiltrated with HR-negative bacteria was cloned by an RT-PCR based 3' RACE (rapid amplification of cDNA ends) method (Frohman 1990). RNA was isolated from inoculated and non-

*Corresponding author. E-mail: zboz@nki.hu

inoculated leaf tissues. 2,5 µg total RNA used in 20 µl for cDNA synthesis. Reverse transcription was initiated with oligot (dT) consisting of a 17-base adapter sequence. The synthesized cDNA was treated with RNAse H.

PCR was performed with Taq polymerase, peroxidase specific and adapter primers for 40 cycles. 2.5 µl cDNA samples were used in 25µl PCR mixtures. The annealing of primers was carried out at 55ºC. The PCR products were separated in 2% agarose gel. The desired fragments were isolated from the gel, cloned into pGEM (Stratagene) vector, and sequenced.

RT-PCR

RNA isolation and cDNA synthesis were carried out as described above. The RNA quality and equivalent amount of different samples were checked by agarose gel electrophoresis and amplification of a permanently expressed actin gene. PCR of peroxidase was performed with tpoxN1 specific primers for 25 cycles. Annealing temperature was 61ºC.

Results and Discussion

In our previous study we have shown that in tobacco leaves infiltrated either with HR-inducing *P. syringae* 61, its HR-negative *hrcC* mutant or with saprophytic *Pseudomonas fluorescens*, at least two new peroxidase isoenzymes appeared in the intercellular washing fluid of leaves (Bozsó et al. 2001). Because these peroxidase isoenzymes seem to be a good markers for detailed study of EIR we tried to clone them. A 3'RACE method as a differential display method was used to isolate induced peroxidase genes. Peroxidase-specific degenerate primers were designed from tobacco sequences found in databases. The size of 3 'RACE products of various peroxidases may be different because the length of coding and mainly the 3' untranslated region of distinct cDNA's varied. For cDNA production samples were collected from leaves injected with HR-negative bacteria (*P. syringae hrcC* mutant or *P. fluorescens*) or control (non-injected or water injected) leaves After agarose gel separation of the amplification products one additional band was found in bacterial injected leaves. The cloned and sequenced band proved to be a previously described tpoxN1 gene. Tpoxn1 was isolated originally from tobacco mosaic virus (TMV) infected tobacco leaves (Hiraga et al. 1999). As it was mentioned above, at least two new peroxidase protein bands were observable in native PAGE. Our failure to clone other activated peroxidase(s) may be due to the fact that the designed primer did not suit amplification of the other peroxidase(s) gene or the other peroxidase(s) is regulated post-transcriptionally or it is also possible that our method was not sensitive enough to detect a low copy number transcript.

With sequence specific primers of tpoxN1 we checked the transcription of the gene in different plant-bacterial interactions. The transcript of tpoxN1 was activated by both the HR-negative *hrcC* mutants of pathogenic *P. syringae* 61 or non-pathogenic *P. fluorescens*, but interestingly, not by the living compatible pathogen *P. syringae* pv. *tabaci*. It seems that the living *P. syringae* pv. *tabaci* actively inhibit the tpoxN1 transcription, because antibiotic killed bacteria induced this gene. The gene was slightly activated in water infiltrated tissues but not in non injected control. These results support the earlier observation that this gene also was transcribed after wounding (Hiraga et al. 2000). We have also tested the tpoxN1 transcript accumulation in virus (TMV) infected leaves and found that tpoxN1 transcription was increased in incompatible, but not, or only slightly in compatible hosts. It is interesting that the HR-negative bacteria and necrosis inducing TMV induce the same gene and maybe the same signaling system and suggests that the induction of tpoxN1 gene is not connected directly to plant cell death.

The role of the peroxidases in plant-bacterial interactions is not clear. There is some evidence that peroxidases accumulate at the site of the bacterial attachment in plant cells and around the bacterial cell. One feature of certain peroxidases is that both can decompose and produce hydrogen-peroxide. The locally accumulated H_2O_2 may directly damage bacteria. Another possibility is that the peroxidases are involved in plant cell wall strengthening at the attachment site by promoting the cross-linking of specific proteins or lignification. This may inhibit the pathogen bacteria to inject gene products into plant cells, or isolate bacteria from the environment, *e.g.* from nutrients.

The peroxidase accumulation in bacterially infected tissues seems to be a widely distributed feature of the plants because it has been described in other species as well (Bestwick et al. 1995; Brown et al. 1998). Our previous results have shown that some peroxidase genes were also activated in Arabidopsis leaves infected with HR-negative bacteria. Detailed characterization of the transcription regulation of these and other genes that were activated during EIR may lead to a better understanding of this interesting and important but little known defense response (EIR).

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