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Gene Note

A novel basic pathogenesis-related protein from tobacco plants

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Abstract Tobacco genes regulated during the hypersensitive response (HR) to infection by tobacco mosaic viruses were screened by differential display and one particular clone with a rapid expression profile was selected and further characterized. The obtained cDNA encoded a polypeptide of 205 amino acids with an apparent molecular mass of 23 kDa and a pI of 10.37 and production of a GFP fusion protein revealed localization in the cytoplasm. Transcripts of the clone were strongly induced in leaves 3 h after HR onset, and 10 min after mechanical wounding, as well as by treatment with jasmonic and salicylic acids. The induction profile satisfies the definition of pathogenesis-related (PR), and the protein was designated as NtPRp23. Database searches indicated the presence of similar proteins in *Arabidopsis* and rice, but with functions yet to be assigned. Our results point to the existence of a novel PR protein family in tobacco plants.

Key words: Hypersensitive response, *Nicotiana tabacum*, PR proteins, wounding.

When attacked by pathogens, plants quickly activate a set of genes that are involved in defense reactions, including signal transduction, gene activation, protein transportation, and production of defense materials and toxicants against the invaders. In the case of resistant tobacco plants, one such reaction is called the hypersensitive response (HR), which occurs upon viral infection (Heath 2000). Infected regions collapse and give rise to a necrotic lesion, thereby restricting pathogens and preventing systemic damage (Hammond-Kozack and Jones 1997). However, the molecular mechanisms underlying the HR are not yet completely understood. In order to identify genes playing critical roles in the HR, we screened for transcripts accumulating immediately after the onset of HR by tobacco mosaic virus (TMV) infection (Yoda et al. 2002; Sugimoto et al. 2004). The genes identified thereby were designated as *HRR* (hypersensitive response related) with serial numbers. In the present study, we focused on one particular clone, *HRR 6*, whose transcripts rapidly accumulated not only upon HR induction but also through mechanical injury. Based on the results described below, we conclude the protein, designated as NtPRp23, to be a novel member of a pathogenesis-related family.

Healthy tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown in a growth cabinet at 23°C under a 14/10 h light/dark photo cycle (Yoda et al. 2002) and mature leaves were detached, inoculated with or without TMV (10 μg/ml^{-1}) using carborundum (Mesh 600), and incubated at 30°C for 48 h, then at 20°C, allowing plants to undergo HR under continuous light exposure. Mechanical wounding was achieved by cutting leaves with a pair of scissors (Hara et al. 2000). For phytohormone treatment, petioles of detached leaves were soaked in 1 mM ethephone, 50 μM jasmonate methyl (MeJA) or 50 μM salicylic acid (SA) solution at 25°C. Treated leaves were harvested after appropriate time intervals, immediately frozen in liquid nitrogen and stored at −80°C until use for further experiments. Southern and northern hybridizations were essentially performed as described earlier (Yoda et al. 2002). For this purpose, 20 μg samples of total RNA were isolated by the AGPC method (Chomczynski and Sacchi 1987) and fractionated by formaldehyde/agarose gel electrophoresis. Total DNAs were prepared as described, fractionated by agarose gel electrophoresis (Hara et al. 2000), transferred to nylon membranes (Hybond-N, Amersham) and subjected to hybridization with appropriate ^{32}P-labeled probes. Expression of GFP fusion proteins in spinach cells was carried out as described (Yoda et al. 2002). Briefly, a plasmid containing the entire coding region of *NtPRp23* from the original λZAPII phage library was cut out with *Xba*I and *Bam*HI, and introduced into the *Xba*I/*Bam*HI site of the pGTBH1-GFP vector, creating an in-frame fusion

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The sequence reported in this article has been deposited in the GeneBank database under accession number AB024512.

Abbreviations: GFP, green fluorescence protein; HR, hypersensitive response; JA, jasmonic acid; PR, pathogenesis-related; SA, salicylic acid; TMV, tobacco mosaic virus.

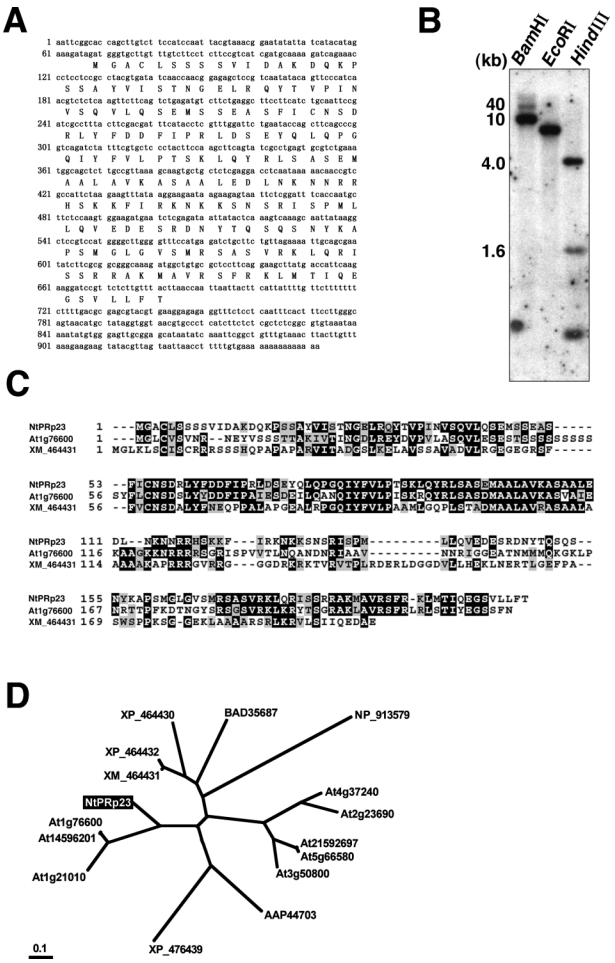

Figure 1. Properties of *NtPRp23*. (A) Nucleic and deduced amino acid sequences for *NtPRp23*. Deduced amino acids for the ORF are shown under the nucleic acid sequence. (B) Southern-blot analysis of *NtPRp23*. A 20 μg aliquot of genomic DNA from *N. tobacum*, cv Xanthi NN was digested with the indicated restriction enzyme, fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with radioactively labeled *NtPRp23* as a probe. (C) Amino acid sequence comparison for *NtPRp23*. Multiple alignment was performed with putative related proteins from *Arabidopsis* (At1g76600) and rice (XM_464431). (D) Phylogenetic tree showing the relationship between *NtPRp23* and other proteins. Amino acid sequences were aligned using the ClustalW program and the tree was constructed with Tree View software. Sequences were obtained from the Database under the indicated accession numbers.

product between the coding region and GFP under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Spinach cells were transformed by particle bombardment as described (Yoda et al. 2002) with gold particles (Bio Rad) coated with the resulting plasmid, incubated at 25°C in darkness for 6 h and viewed under an epifluorescence microscope (Ax70, Olympus, Tokyo).

The full length cDNA for *NtPRp23* was 1046 bp and the deduced polypeptide consisted of 205 amino acids with an apparent molecular mass of 23.1 kDa and a pI of 10.37 (Figure 1A). Genomic Southern analysis showed rather simple hybridization patterns upon digestion with several restriction enzymes (Figure 1B). Although

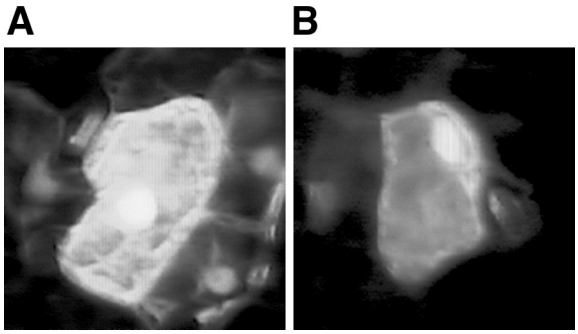

Figure 2. Cellular localization of *NtPRp23*. Spinach leaf cells were transformed with plasmids expressing *GFP* alone (A) or the *NtPRp23::GFP* fusion protein (B). Green fluorescence under UV illumination was assessed by epifluorescence microscopy.

interpretation of such data is generally confounded by the amphidiplod nature of *N. tabacum*, the observed hybridization patterns suggested that *NtPRp23* exists as a single copy gene in each chromosomal set of this plant. A homology search identified similarities with putative proteins from *Arabidopsis* (At1g76600) and rice (XM_464431), indicating the existence of a protein family among higher plants (Figure 1C). Indeed, screening of *Arabidopsis* and rice databases with *Arabidopsis* clone At1g76600 and rice XM_464431 showed that there are at least 8 and 5 similar genes in each plant, respectively. A phylogenetic analysis showed that they form a small family, in which *NtPRp23* is close to *Arabidopsis* proteins (Figure 1D).

Cellular localization of the *NtPRp23* protein was examined with *GFP*-fused constructs. A plasmid containing the CaMV 35S promoter *NtPRp23::GFP* gene was biolistically bombarded into spinach cells and epifluorescence analyses indicated the control construct containing only *GFP* to show signals throughout cells, including the cytoplasmic space (Figure 2). Cells with the *NtPRp23::GFP* construct also showed similar fluorescent signals, suggesting *NtPRp23* localization in the cytoplasm (Figure 2).

Transcript accumulation of *NtPRp23* was then analyzed. In unstressed healthy plants, transcripts were not observed in upper ground tissues, leaves, stems or flowers, whereas they were constitutively accumulated in root tissues, and to a lesser extent, in cultured BY2 cells (Figure 3A). Based on this observation, effects of diverse treatments on transcript accumulation were examined. When tobacco plants carrying the resistant *N* gene are inoculated with TMV and incubated at 30°C, at which the *N* gene does not function, viral particles multiply. Upon transfer to 20°C, the *N* gene is activated, resulting in lethal HR. *NtPRp23* transcripts began to accumulate as early as 1 h after this temperature shift, reaching a maximum after 3 h and declining thereafter (Figure 3B). Accumulation was observed not only in inoculated local leaves but also in non-inoculated adjacent leaves,

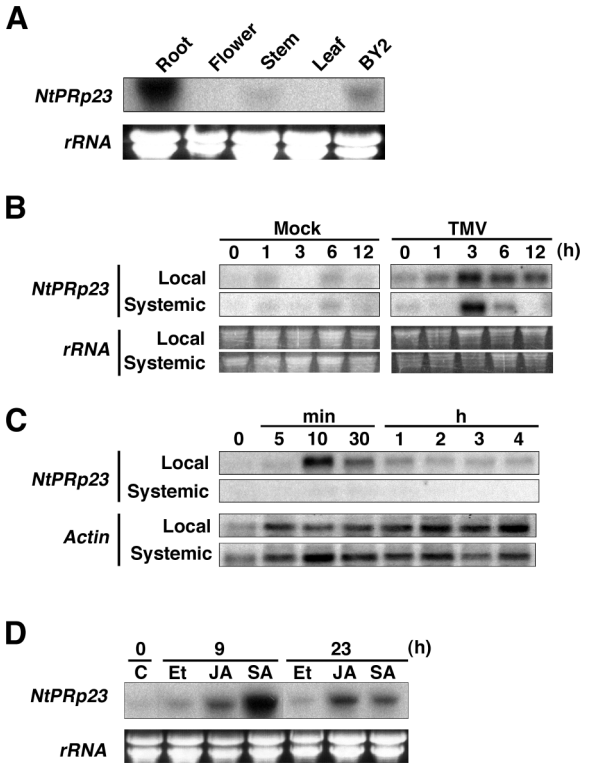

Figure 3. Accumulation profile of *NtPRp23* transcripts. (A) Tissue-specificity. A 20 μg aliquot of total RNA from the indicated tissue was fractionated by formaldehyde/agarose gel electrophoresis, transferred to a nylon membrane and subjected to RNA blot hybridization with a radioactively labeled *NtPRp23* probe. (B) Effects of TMV infection. Detached healthy leaves from *N. tabacum*, cv Xanthi NN were treated with 10 mM sodium phosphate buffer alone (Mock), or containing TMV (10 μg/ml) (TMV), incubated at 30°C for 48 h, and then transferred to 20°C. Total RNA was isolated at the indicated time points, and 20 μg aliquots per lane were assayed as described above. (C) Effects of wounding. Healthy intact leaves were mechanically wounded by cutting with a pair of scissors, harvested at the indicated time points and total RNA was analyzed as described above. (D) Effects of SA and MeJA. Detached healthy leaves of *N. tabacum*, cv Xanthi NN were left for 4 h for acclimatization to the initial wound stress, then treated with 50 μM SA (SA), 50 μM MeJA (JA) or 100 μM ethephon (Et). The control was an untreated sample (C). Total RNA was isolated at the indicated time points, and 20 μg of total RNA per lane was fractionated and hybridized as described above. As the loading control, rRNA or actin cDNA was used (lower panel).

indicating transcriptional activation to have occurred systemically. Mechanical wounding also induced a temporal transcript accumulation within 10 min (Figure 3C). In contrast to HR, however, wounding responses occurred only locally. When leaves were treated with MeJA and SA, transcript accumulation was seen at 9 h with a gradual decline by 23 h (Figure 3D). Ethephone exerted almost no effects (Figure 3D).

The finding that accumulation of *NtPRp23* transcripts increased on TMV infection and mechanical wounding provided a profile that satisfies the definition of PR proteins by van Loon et al. (1994). In addition, *NtPRp23* clearly responded to JA and SA, which serve as wound and pathogen signals, respectively, and activate many genes encoding PR proteins (Ohashi and Ohshima 1992). The organ-specific expression and induction patterns of *NtPRp23* are also similar to other basic PR proteins as exemplified by tobacco glucanases (van de Rhee et al. 1993). It was thus concluded that *NtPRp23* should be considered as a novel member of the PR protein family.

Pathogenesis-related proteins were originally identified in plants upon viral infection, and are typically classified into five groups, the PR-1 to PR-5 proteins (van Loon 1985). In tobacco plants, however, additional PR-like proteins have been found, and currently at least 11 groups are registered (van Loon et al. 1994). Although some functions have been proposed, physiological roles of the majority of them have yet to be determined. Further functional analysis of *NtPRp23* may provide additional information on PR-protein research.

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