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Dark-induced accumulation of a basic pathogenesis-related (PR-1) transcript and a light requirement for its induction by ethylene

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Abstract

A gene encoding a basic-type pathogenesis-related protein from *Nicotiana tabacum* (*prb*-1b) was cloned, sequenced and characterized. It contains an open reading frame of 179 amino acids that is ca. 65% homologous with the acidic PR-1 class of pathogenesis-related proteins and 87% homologous with a different basic-type PR-1 gene. In the light, physiological levels of ethylene rapidly (1 h) induced basic, but not acidic-type, PR-1 transcript. Additional elicitors acting via ethylene, such as α-aminobutyric acid, were shown to induce basic- and acidic-type PR-1 transcript accumulation in a light-dependent manner. In contrast, xylanase, an ethylene-independent elicitor, induced transcript accumulation of basic- and acidic-type PR-1 in a light-independent manner. Dark-induced accumulation of basic PR-1 transcript occurred at night in greenhouse-grown plants and, to a greater extent, in continuously dark-treated plants. The novel dark regulation may point to additional nonpathogenesis-related roles for these genes in plant-environment interactions.

Introduction

Pathogenesis-related (PR) proteins were initially defined as a group of acidic-type proteins that accumulate in the extracellular space of TMV-infected leaves of hypersensitively reacting tobacco plants [3, 35]. Functions were assigned to some of these proteins such as endoglucanases (PR-2a, b and c [14]) and endochitinases (PR-3a and b [17]). Other PR proteins have suspected functions based on sequence homology. One of them, PR-5, is homologous to a bifunctional α-amylase/trypsin inhibitor from maize [30] and to an osmotic stress-inducible tobacco protein termed osmotin [32]. In contrast, the PR-1 protein family has no assigned biological function and no significant homology to any plant gene. Very limited homology exists to a mammalian sperm-coating glycoprotein [4], to a mammalian testis specific protein [13], and to the white-face hornet venom allergen [9].

Serological and molecular comparisons revealed that most acidic-type PR proteins have basic-type homologues as well [3, 8]. These proteins differ in some cases from their acidic-type counterparts in their induction pattern [24, 37] and, in the case of basic glucanases, in their intracellular distribution [15, 33]. The isolation and

sequence of a basic-type PR-1 cDNA [34] and a basic-type PR-1 genomic clone have been reported [28]. They appear, in a fashion similar to their acidic-type counterparts, to be encoded by a gene family of up to 8 members [6]. Studies involving the expression of the basic-type PR-1 cDNA revealed that the transcript accumulated in virus-infected or ethephon-treated leaves, in roots and in leaves of axenically cultured plants [23, 24].

The induction of acidic-type PR proteins has been shown to occur through at least two separate signal transduction pathways [10, 20, 21, 36]. One induction pathway is ethylene-dependent in its mode of action and is exemplified by the elicitors, TMV and α-aminobutyric acid. This was demonstrated by the abrogation of their ability to elicit acidic-type PR-1 protein accumulation in the presence of inhibitors of ethylene biosynthesis or action [21].The other induction pathway, exemplified by the use of the elicitor xylanase, does not require ethylene. The ethylene-dependent induction was further characterized as requiring light, while ethylene-independent induction did not. The existence of a basic-type PR gene that is directly responsive to ethylene offers an opportunity to further characterize these pathways. Here we report the isolation, characterization, and unique dark-induced expression of a basic-type PR-1 gene.

Materials and methods

Plant material

Nicotiana tabacum cv. 'Samsun NN' plants were grown in the greenhouse, in 16 h day/8 h night diurnal cycles. Experiments in the light were conducted in the greenhouse on young potted plants with four to five leaves of at least 10 cm length.

Induction of PR transcripts in leaves

Induction by TMV was achieved by applying TMV (U2 strain) at an inoculum concentration that yielded 200 lesions per leaf [21]. Chemical induction was achieved by spraying leaves until run-off with a solution of either 5 mM acetylsalicylic acid, 7 mM ethephon or 5 mM α-aminobutyric acid. All solutions contained 0.01% Tween 20. Induction by purified xylanase was achieved by injecting a solution of 10 ng/ml into leaves as described [21]. For ethylene treatments a constant stream of air containing 18 μl/l ethylene was applied to a sealed glass box housing the potted plants. Light was provided by a mixture of 'Cool White' and 'Grolux' fluorescent lamps (25–30 $\mu E\ m^{-2}\ s^{-1}$). Nocturnal measurements of transcript were carried out in the greenhouse. The background light measured at night was less than 0.05 $\mu E\ m^{-2}\ s^{-1}$. Extended dark treatments were in dark chambers with constant air exchange.

RNA isolation and northern blot hybridization

Total RNA was isolated [19] from leaves frozen in liquid nitrogen and stored at −80 °C. Twenty μg of total RNA were fractionated in a formaldehyde denaturing gel and transferred to nitrocellulose [31]. Blots were hybridized to nick-translated acidic-type or basic-type PR-1 DNA probes at 50 °C, 50% formamide. Under these relatively stringent hybridization conditions no cross-hybridization between basic-type and acidic-type PR-1 was detected. The basic-type PR-1 probe was *prb*-1b. The acidic-type PR-1 probe was an undescribed, expressed, gene 94% homologous to PR1-b (Y. Eyal, unpublished).

Construction and screening of a genomic library

Nicotiana tabacum cv. Samsun NN genomic DNA was used to prepare a genomic library in phage λ-EMBL3. Primary recombinants (1×10^6) were amplified to create a stable library. Plaques (4×10^5) were screened using Colonyscreen membranes (NEN). Positive clones were plaque-purified by two more screening rounds.

Sequencing and primer extension analysis

Phage inserts were subcloned into Bluescript plasmids (Stratagene) and sequenced by the dideoxy chain termination technique using Sequenase (USB). For primer extension, a synthetic end-labelled oligonucleotide (5′-TGTCCCATG-TCATGGGA-3′) corresponding to nucleotide position 131–147 (Fig. 1) was used to prime poly(A)-enriched RNA isolated from TMV-induced tobacco leaves. Primer extension was carried out as previously described [31].

Results

Isolation of a basic-type PR-1 genomic clone and sequence analysis

A genomic library was constructed from DNA of *Nicotiana tabacum* cv. Samsun NN and screened

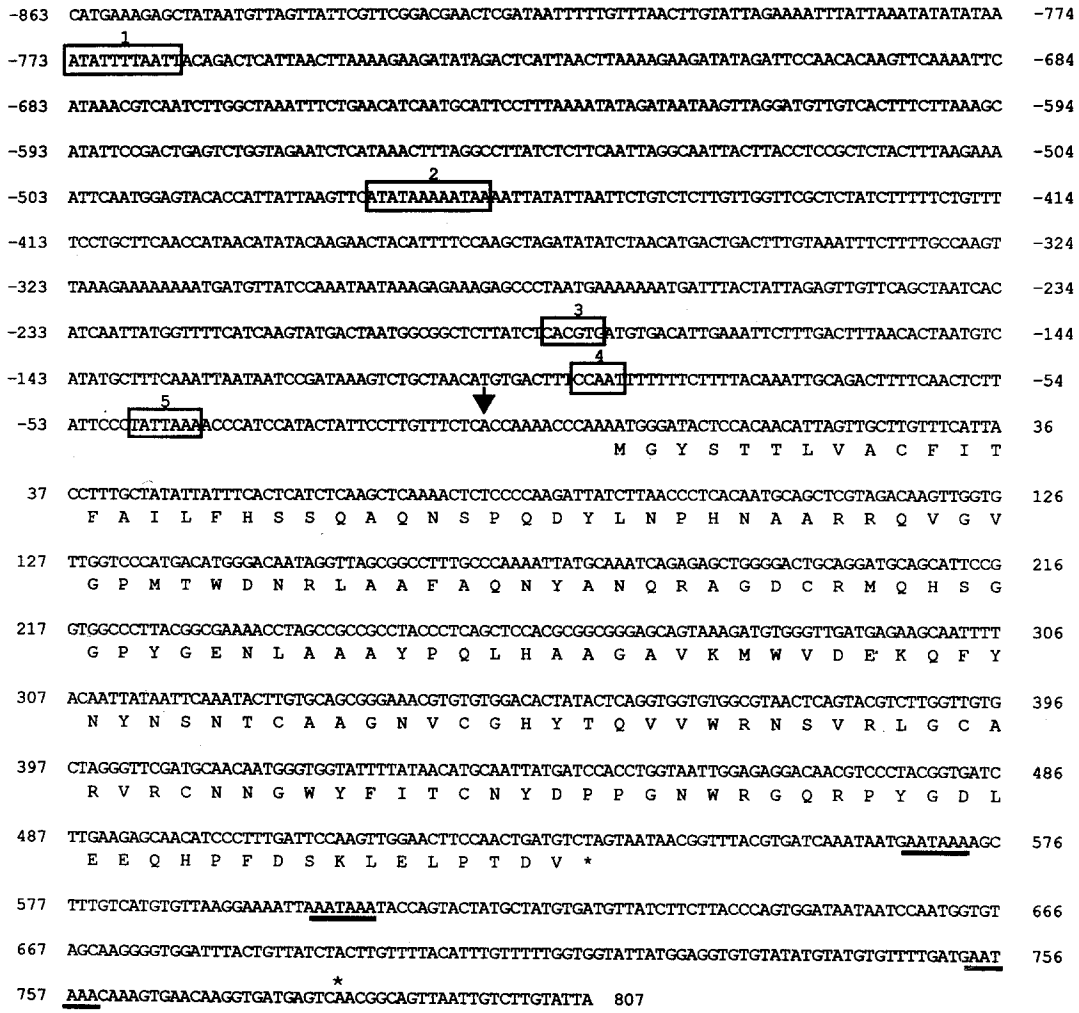

Fig. 1. Nucleotide sequence of *prb*-1b. A decoded open reading frame of 179 amino acids is shown in one-letter code. The upstream region of the gene features two AT-1 [7] like sequences (boxes 1 and 2), a G-box [11] consensus core sequence (box 3) and putative CAAT (box 4) and TATA (box 5) boxes. Transcription initiation (denoted by arrow) occurs 12 bp upstream of the translation initiation codon. Polyadenylation signals (underlined) occur at three sites downstream of the translational stop codon. The star at nucleotide 783 denotes the transcription termination in the previously published cDNA sequence [34].

using an acidic-type PR-1 genomic clone as described in Materials and methods. Low-stringency screening yielded a basic-type PR-1 clone, the sequence of which appears in Fig. 1. Henceforth, this gene will be referred to as *prb*-1b to conform with suggested nomenclature conventions [25]. '*b*' identifies it with the basic-type subclass of PR-1 genes and 'b' differentiates it from a previously isolated basic-type PR-1 gene characterized thus far only by sequence, which will be henceforth termed *prb*-1a [28]. The open reading frame codes for a 179 amino acid protein which shares 87% homology with the deduced amino acid sequence of *prb*-1a [28], and 64% homology with the acidic-type protein PR-1b ([5, 29], Fig. 2). At the nucleotide sequence level the *prb*-1b transcribed region appears to be identical, in the coding and noncoding regions, to a previously published basic-type PR-1 cDNA [34]. However, a sole difference, an additional 'C' present in *prb*-1b at position 476, results in a shift in the reading frame relative to the published basic-type PR-1 cDNA sequence [6]. The predicted amino acid sequence of the carboxy terminus of the basic-type PR-1 cDNA which lacks this C is both longer and at variance with *prb*-1b and with *prb*-1a ([28], Fig. 2). While the previously published cDNA may be encoded by an additional gene of the *prb*-1 subclass, it is most likely an RNA product transcribed from *prb*-1b. We have detected sequence compression artifacts in that region, which may account for the discrepancy.

The *prb*-1b upstream promoter region is ca. 70% homologous to the *prb*-1a upstream region (allowing for large gaps in the comparison) up to position −654 where homology abruptly stops (not shown). Homology with the upstream region of acidic-type PR-1 genes is very low and limited to several boxes in the first 150 bases of the promoter [8]. Several sequences resembling putative nuclear protein binding sites were found in the *prb*-1b upstream region (Fig. 1). The most outstanding of these include a G-box core motif [11] and two AT-1 like sequences [7].

Primer extension analysis was performed using a gene specific oligonucleotide (Fig. 3). It localized transcription initiation to a sequence resembling the consensus sequence for initiation, at a point 12 bp upstream of the translation initiation codon, resulting in a relatively short RNA leader sequence [12]. An additional fainter band migrating at 1 bp slower mobility, presumably indicates transcription initiation from a different gene in this subfamily. Indeed, the distance between the primer binding site and the presumed transcription initiation site present in *prb*-1a is also greater by one base relative to *prb*-1b. Thus the additional primer extension product is consistent with

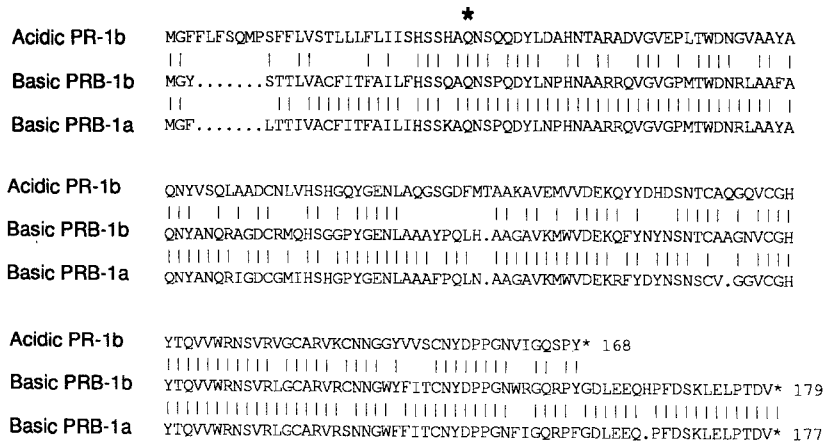

Fig. 2. Comparison of acidic-type PR-1 and basic-type *prb*-1 deduced amino acid sequences. Spaces were integrated into the sequences to allow for maximum alignment. Match lines indicate sequence homologies to *prb*-1b. The amino acid sequence length of each open reading frame is indicated at the end of the sequence. The star denotes the first amino acid of the mature acidic PR-1 polypeptide.

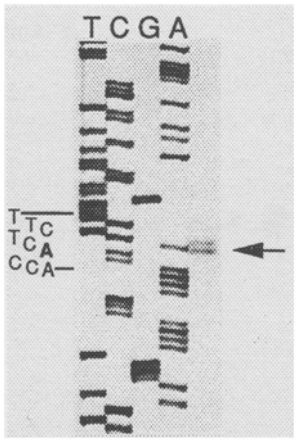

Fig. 3. Autoradiograph of primer extension analysis of basic *prb*-1b mRNA. An oligonucleotide complementary and specific for *prb*-1b transcript was used to prime the reverse transcription of leaf mRNA isolated from TMV inoculated leaves (right lane). The same oligonucleotide was used to prime nucleotide sequence reactions from the appropriate cloned DNA (lanes marked TCGA). Arrow and bold letter denote *prb*-1b transcription initiation site.

primer extension originating from a putative *prb*-1a transcript.

Differential expression pattern of basic-type and acidic-type PR-1

The induction of basic isoforms of PR-1 genes was shown to involve ethylene, with basic PR-1 transcripts accumulating 1–2 days after ethephon treatment [24]. The use of an artificial ethylene precursor precludes the estimation of ethylene dose and time sequence of induction. We therefore exposed plants to a constant stream of hormone-like physiological concentrations of ethylene (18 μl/l) in the light and examined plants for *prb*-1 transcripts. Based on densitometric measurements, *prb*-1 transcripts increased 3-fold within 1 h and accumulated 100-fold after 6 h of exposure to ethylene (Fig. 4A). The hybridization results represent the total accumulation of basic-type *prb*-1 gene family transcripts and do not differentiate between genes within this family. Under these conditions little or no induction of acidic-type PR-1 transcript was detected (Fig. 4B).

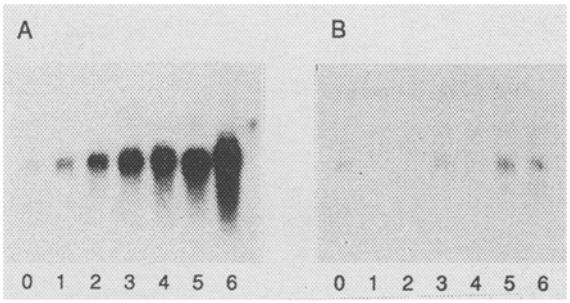

Fig. 4. Northern analysis of RNA from leaves treated by ethylene in the light. Each lane contains 20 μg of total RNA extracted from leaves treated with 18 μl/l ethylene from 0 to 6 hours and hybridized with *prb*-1b probe in panel A and with the acidic PR-1 probe in panel B.

Basic-type and acidic-type PR-1 genes were found to be induced after infection by TMV. In both cases accumulation of transcript began between 24 and 48 h after inoculation (Fig. 5, panels A and E). Salicylic acid and ethephon were relatively rapid inducers of *prb*-1 (Fig. 5, panels B, C, F and G). However their inductive effect was differential. Ethephon induced the expression of the basic-type *prb*-1 genes exclusively (Fig. 5, panels C and G), while salicylic acid induced both types of genes (Fig. 5, panels B and F). Accumulation of *prb* 1 transcripts began ca. 8 h after ethephon treatment and reached a peak 12 h after treatment, followed by a gradual decrease in transcripts abundance. The delayed response in transcript accumulation relative to ethylene (Fig. 4) may reflect the additional time that it takes for ethephon to penetrate the leaf and evolve into ethylene. Repeated ethephon application and interminable ethylene exposure induced stable elevated transcript levels (data not shown). Application of α-aminobutyric acid was shown to cause ethylene evolution and induce microlesions on tobacco leaves [21]. It was an efficient inducer of both *prb*-1 and acidic PR-1 transcripts (Fig. 5, panels D and H).

Dark-induced accumulation of basic-type PR-1 transcripts

Low transcript levels present in untreated plants collected during early hours of the morning caused

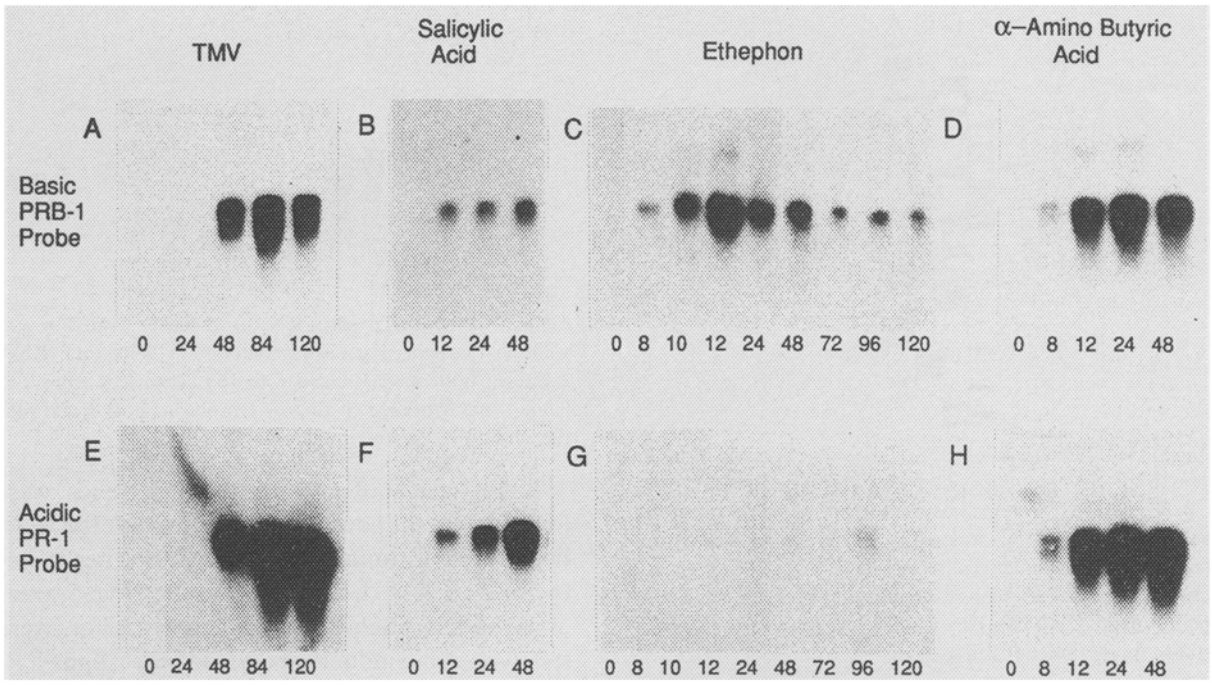

Fig. 5. Northern analysis of differential induction of basic-type *prb*-1 and acidic type PR-1 transcript accumulation. Each lane contains 20 μg of total RNA extracted from leaves inoculated with TMV (panels A, E); sprayed with 5 mM acetylsalicylic acid (panels B, F); sprayed with 7 mM ethephon (panels C, G); sprayed with 5 mM α-aminobutyric acid (panels D, H). Blots in panels A–D were hybridized with *prb*-1b probe. Blots in panels E–H were hybridized with acidic PR-1 probe. Numbers in panels indicate hours after treatment.

us to suspect a dark effect on *prb*-1 gene expression. When untreated green-house plants were sampled over a 24 h period, we found that the basal level of *prb*-1 transcript had a day/night cycle (Fig. 6). During daytime the transcript in untreated plants was undetectable. Several hours of 'natural' darkness were sufficient for the accumulation of detectable, albeit low, levels of transcript. Experiments involving elicitor induction were therefore initiated at noon time. To further substantiate the phenomenon of dark-induced accumulation of *prb*-1 transcript, greenhouse plants were transferred to extended complete darkness for up to 72 h and were then returned to the light. *prb*-1 transcript accumulated in the dark and reached maximum levels of 50-fold induction, based on densitometric measurements, after 48 h (Fig. 7). The steady state dark-induced accumulation was 30% of the maximum achieved

Fig. 6. Northern analysis of diurnal cycle of basic *prb*-1 transcript accumulation. Each lane contains 20 μg of total RNA extracted from greenhouse plants. Leaves were sampled at noon (time = 12), midnight (time = 24, 3 hours of darkness) and at 02.00 (time = 02, 5 hours of darkness). The RNA blot was hybridized using the *prb*-1b probe.

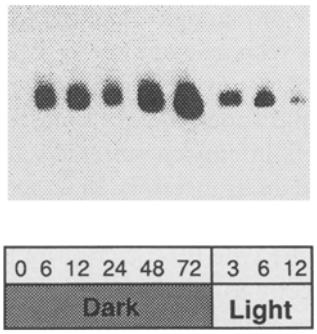

Fig. 7. Northern analysis of dark-induced accumulation of basic *prb*-1 transcript. Greenhouse plants were transferred to a darkroom for up to 72 h after which they were returned to the greenhouse for 12 additional hours. Each lane contains 20 μg of total RNA extracted from leaf samples taken at the hours indicated. The RNA blot was hybridized with the *prb*-1b probe.

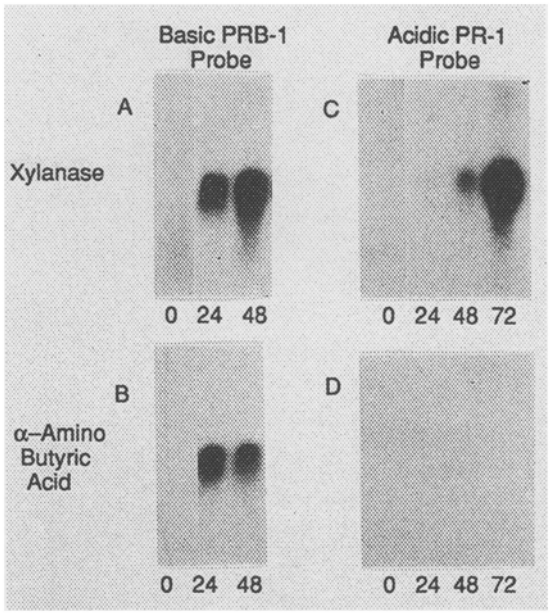

Fig. 8. Northern analysis of elicitor induced accumulation of acidic-type PR-1 and basic-type *prb*-1 transcripts in the dark. Plants were incubated in the dark for 24 h prior to treatment with either xylanase, panels A, C or α-aminobutyric acid, panels B, D. Leaf samples were taken at the onset of darkness (time = 0). One day later, in the dark, elicitor treatment commenced (time = 24). Leaves were sampled 24 and 48 hours after elicitor treatment (time = 48 and 72). Each lane contains 20 μg of total RNA. The RNA blots were hybridized to either the basic-type *prb*-1 probe (panels A, B) or the acidic-type PR-1 probe (panels C, D).

by induction in light with α-aminobutyric acid when northern analysis was carried out coincidentally. Decrease of the relatively high transcript levels occurred following the return of plants to the light, so that after 12 h the transcript level was barely detectable. Dark-induced accumulation of transcript was not detected in the acidic-type PR-1 family (data not shown).

Light requirement of the ethylene-dependent signal transduction pathway

Light has been shown to be necessary for induction of acidic-type PR proteins via the ethylene dependent signal transduction pathway [21]. A second induction pathway, which does not require ethylene, was shown to be light-independent [21]. Therefore, the differential effect of light on these induction pathways was examined for acidic-type PR-1 and basic-type *prb*-1 transcripts. Plants were adapted for 24 h in the dark prior to treatment with elicitors. The elicitor, α-aminobutyric acid via ethylene (Fig. 8, panel B), did not induce the basic-type *prb*-1 transcript in the dark above the basal dark level (compare 48 h dark treatment to 24 h dark treatment). Similarly, the application of α-aminobutyric acid did not induce accumulation of acidic-type PR-1 transcript in the dark (Fig. 8, panel D) although it was an efficient inducer in the light (Fig. 5, panels H). While the ethylene independent elicitor xylanase appeared to enhance the level of basic-type *prb*-1 transcript in the dark (Fig. 8, compare panels A and B), the dark-induced basal level of *prb*-1 makes accurate estimation of this accretion difficult. However, clearly xylanase, in contrast to α-aminobutyric acid, was capable of inducing transcript accumulation of acidic-type PR-1 in the dark (Fig. 8, compare panels C and D). To unequivocally show that ethylene mediated induction requires light, plants were treated with physiological levels of exogenous ethylene in the dark and in the light (Fig. 9). Accumulation of *prb*-1 transcript after 6 h in the light was 5-fold more than that obtained in the dark (compare Fig. 9, panels A and B and the accompanying

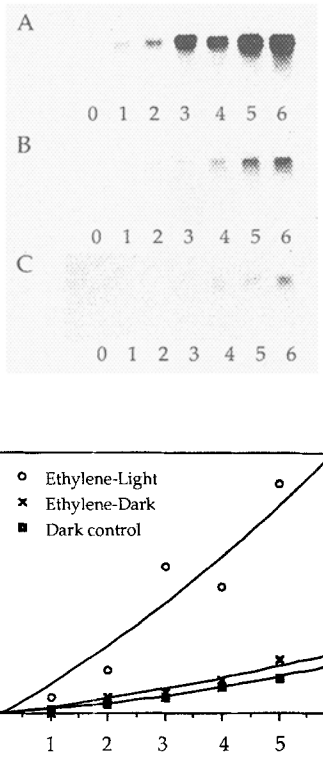

Fig. 9. Northern analysis of RNA from ethylene-treated leaves maintained in the light (A) or dark (B) or without ethylene in the dark (C). Each lane contains 20 μg of total RNA extracted from leaves treated with or without 18 μl/l ethylene for 0 to 6 h. The blots in A–C were hybridized together with the *prb*-1b probe. A densitometric scan of the results in panels A–C is shown in panel D.

graph in panel D). The application of ethylene to leaves in the dark did not significantly enhance dark-induced accumulation of *prb*-1 transcript (compare Fig. 9, panels B and C, and the accompanying graph in panel D).

Discussion

The coordinated accumulation of the various PR protein classes in response to pathogens might give the impression, albeit misleading, of a common pathway for the induction of all PR protein genes. However, analysis of induction by different elicitors and the influence of light reveal a complex expression pattern. *prb*-1 can be directly induced by ethylene. Indeed the rapid accumulation of *prb*-1 transcript in the presence of hormone-like physiological quantities of ethylene is the fastest response shown by PR genes with any elicitor. The time sequence of events is similar to the rapid appearance of senescence-related gene products in ethylene-treated carnation petals [16] and of transcripts that accumulate in ethylene-treated green tomato fruit [18]. In contrast, the acidic-type PR-1 transcript level did not respond directly to ethylene, although it was induced with α-aminobutyric acid which depends on ethylene evolution for elicitation [21]. Hence, ethylene is necessary but not sufficient for induction of acidic-type PR-1 expression. The data are consistent with a dual signal requirement for induction of acidic PR-1 via ethylene dependent elicitation. One component required is ethylene, as inhibitors of ethylene biosynthesis and action obstruct this induction pathway [21]. The nature of the second component is unknown. Thus, even closely related genes in the PR-1 family show differential induction and alternative mediators exist in the interaction between pathogens and plants.

Light-dependent induction of acidic-type PR proteins has been previously noted [1, 20, 21]. Here we confirm and extend this observation to measurement of transcript accumulation of basic and acidic-type PR-1 genes. The light requirement is not an inherent character of the genes, but apparently influences the signal transduction pathway. The elicitor, α-aminobutyric acid, which acts through the ethylene-requiring pathway did not induce acidic- or basic-type PR-1 transcript accumulation in the dark. In contrast, xylanase, an ethylene-independent elicitor, was able to induce acidic-type PR-1 and seemingly basic-type *prb*-1 transcript accumulation in the dark. We have shown previously that the α-aminobutyric acid elicitation of acidic PR-1 requires light but not active photosynthesis [21]. As α-aminobutyric acid provided a dual signal for acidic-type PR-1 induction, we were unable to assign the site of dark inhibition to ethylene or to the other, as yet, undefined second component. However, the light-dependent responsiveness of *prb*-1 to ethylene leads us to conclude that the light-requiring step occurs somewhere in the ethylene compo-

nent of the signal transduction pathway. The effect of light on ethylene action reported here is not to be confused with its previously reported, opposing, inhibitory effect on ethylene biosynthesis [38]. A light requirement for PR transcript accumulation during ethylene-mediated induction is unexpected, since exogenously applied ethylene was shown to be active in the dark during abscision, fruit ripening and tissue senescence [26]. We note that our experiments are conducted in relatively young leaves and that the light dependence for PR protein induction via ethylene may be limited to this tissue type.

Our results provide the first evidence for direct dark-induced accumulation of a PR protein transcript. Thus, light plays a dual role in PR protein accumulation. The presence of light is necessary to maintain ethylene-dependent signal transduction. In the absence of light basic-type PR-1 transcripts accumulate. The dark-induced accumulation is physiologically significant as it appears during normal diurnal growth cycles. The activity of this gene in the dark suggests a requirement by the plant for *prb*-1 gene product during dark growth that is advantageously enhanced during pathogenesis. It may indicate non-stress-related functions for *prb*-1 as have been proposed for other PR proteins [20, 27]. Recently a senescence-associated gene that showed limited homology to the PR-1 class was reported to be dark-induced [2]. Whether the senescence-related gene is induced by pathogens is not known. The dark accumulation of transcript may involve enhanced RNA stability or direct transcriptional activation. Additional experiments including analysis of chimaeric transgenes should shed more light on this question.

Northern analysis of multigene families cannot discriminate between individual gene members. However, the use of specific oligonucleotide-directed reverse transcription illustrates directly the activity of this gene or a related gene containing the same sequence. In addition, transgenic plants containing 862 bp of upstream promoter region of *prb*-1b fused to the GUS reporter gene exhibit similar results (Y. Eyal, in preparation). A protein product has yet to be described for *prb*-1b. We have recently raised antibodies specific for the basic-type PR-1 protein via generation of an engineered fusion protein in bacteria. Significantly, the antisera detected a plant protein product accumulating in ethylene-treated leaves (Y. Eyal, in preparation). Studies of the expression pattern and cellular localization of the protein will point out parallels and differences between the *prb*-1b protein product and the basic and acidic-type forms of other PR proteins.

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References

1. Asselin A, Grenier J, Cote F: Light-influenced extracellular accumulation of b (pathogenesis-related) proteins in *Nicotiana* green tissue induced by various chemicals or prolonged floating on water. Can J Bot 63: 1276–1283 (1985).
2. Azumi Y, Watanabe A: Evidence for a senescence-associated gene induced by darkness. Plant Physiol 95: 577–583 (1991).
3. Bol JF, Linthorst HJM, Cornelissen BJC: Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopath 28: 113–138 (1990).
4. Brooks DE, Means AR, Wright EJ, Singh SP, Tiver KK: Molecular cloning of the cDNA for androgen-dependent sperm-coating glycoproteins secreted by the rat epididymis. Eur J Biochem 161: 13–18 (1986).
5. Cornelissen BJC, Hooft van Huijsduijnen RAM, Van Loon LC, Bol JF: Molecular characterization of messenger RNAs for 'pathogenesis-related' proteins 1a, 1b and 1c, induced by TMV infection of tobacco. EMBO J 5: 37–40 (1986).
6. Cornelissen BJC, Horowitz J, Van Kan JAL, Goldberg RB, Bol JF: Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. Nucl Acids Res 15: 6799–6811 (1987).
7. Datta N, Cashmore AR: Binding of a pea nuclear protein to promoters of certain photoregulated genes is mod-

ulated by phosphorylation. Plant Cell 1: 1069–1077 (1989).
8. Eyal Y, Fluhr R: Cellular and molecular biology of pathogenesis related proteins. In: BJ Miflin (ed) Oxford Surveys of Plant Molecular and Cellular Biology 7, pp. 223–254. Oxford University Press, Oxford (1991).
9. Fang KSY, Vitale M, Fehlner P, King TP: cDNA cloning and primary structure of a white-face hornet venom allergen, antigen 5. Proc Natl Acad Sci USA 85: 895–899 (1988).
10. Felix G, Meins F Jr: Ethylene regulation of β-1,3-glucanase in tobacco. Planta 172: 386–392 (1987).
11. Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR: An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci USA 85: 7089–7093 (1988).
12. Joshi CP: An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucl Acids Res 15: 6643–6653 (1987).
13. Kasahara M, Gutnecht J, Brew K, Spurr N, Goodfellow PN: Cloning and mapping of a testis-specific gene with sequence similarity to a sperm coating glycoprotein gene. Genomics 5: 527–534 (1989).
14. Kauffmann S, Legrand M, Geoffroy P, Fritig B: Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J 6: 3209–3212 (1987).
15. Keefe D, Hinz U, Meins F Jr: The effect of ethylene on the cell-type-specific and intracellular localization of β-1,3-glucanase and chitinase in tobacco leaves. Planta 182: 43–51 (1990).
16. Lawton KA, Raghothama KG, Goldsbrough PB, Woodson WR: Regulation of senescence-related gene expression in carnation flower petals by ethylene. Plant Physiol 93: 1370–1375 (1990).
17. Legrand M, Kauffmann S, Geoffroy P, Fritig B: Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA 84: 6750–6754 (1987).
18. Lincoln JE, Cordes S, Read E, Fischer RL: Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc Natl Acad Sci USA 84: 2793–2797 (1987).
19. Logemann J, Schell J, Willmitzer L: Improved method for the isolation of RNA from plant tissues. Anal Biochem 163: 16–20 (1987).
20. Lotan R, Fluhr R: Function and regulated accumulation of plant pathogenesis-related proteins. Symbiosis 8: 33–46 (1990).
21. Lotan T, Fluhr R: Xylanase, a novel elicitor of pathogenesis-related proteins in tobacco, uses a non-ethylene pathway for induction. Plant Physiol 93: 811–817 (1990).
22. Lotan T, Ori N, Fluhr R: Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell 1: 881–887 (1989).
23. Memelink J, Hoge JHC, Schilperoort RA: Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. EMBO J 6: 3579–3583 (1987).
24. Memelink J, Linthorst HJM, Schilperoort RA, Hoge JHC: Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol Biol 14: 119–126 (1990).
25. Mundy J: Developing nomenclature for genes of unknown function: A case study of ABA-responsive genes. Plant Mol Biol Rep 7: 276–283 (1989).
26. O'Brien T, Beall FD, Smith H: De-etiolation and plant hormones. In: Pharis RP, Reid DM (eds) Encyclopedia of Plant Physiology New Series Vol 11. Hormonal Regulation of Development III, pp. 282–307. Springer-Verlag, Berlin (1985).
27. Ori N, Sessa G, Lotan T, Himmelhoch S, Fluhr R: A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superclass. EMBO J 9: 3429–3436 (1990).
28. Payne G, Middlesteadt W, Desai N, Williams S, Dincher S, Carnes M, Ryals J: Isolation and sequence of a genomic clone encoding the basic form of pathogenesis-related protein 1 from *Nicotiana tabacum*. Plant Mol Biol 12: 595–596 (1989).
29. Pfitzner UM, Goodman HM: Isolation and characterization of cDNA clones encoding pathogenesis-related proteins from tobacco mosaic virus infected tobacco plants. Nucl Acids Res 15: 4449–4465 (1987).
30. Richardson M, Valdes-Rodriguez S, Blanco-Labra A: A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. Nature 327: 432–434 (1987).
31. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
32. Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch E, Regnier FE, Bressan RA: Characterization of osmotin. A thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol 85: 529–536 (1987).
33. Van den Bulcke M, Bauw G, Castresana V, Van Montagu M, Vandekerckhove J: Characterization of vacuolar and extracellular β(1,3)-glucanases of tobacco: evidence for a strictly compartmentalized plant defense system. Proc Natl Acad Sci USA 86: 2673–2677 (1989).
34. van Kan JAL: Structure and expression of virus-inducible plant genes. Ph.D. thesis, State University of Leiden, Netherlands (1988).
35. Van Loon LC, Van Kammen A: Polyacrylamide disc electrophoresis of the soluble leaf proteins from *N. tabacum* var. 'Samsun' and 'Samsun NN'. Virology 40: 199–211 (1970).
36. Vogeli U, Meins F Jr, Boler T: Co-ordinated regulation of chitinase and β-1,3-glucanase in bean leaves. Planta 174: 364–372 (1988).
37. Ward ER, Payne GB, Moyer MB, Williams SC, Dincher

SS, Sharkey KC, Beck JJ, Taylor HT, Ahl-Goy P, Meins F Jr, Ryals JA: Differential regulation of β-1,3-glucanase messenger RNAs in response to pathogen infection. Plant Physiol 96: 390–397 (1991).

38. Yang SF, Hoffman NE: Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol 35: 155–189 (1984)