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Transcriptional activation of 2 classes of genes during the hypersensitive reaction of tobacco leaves infiltrated with an incompatible isolate of the phytopathogenic bacterium *Pseudomonas solanacearum*

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Abstract

Fourteen cDNA clones whose corresponding mRNAs accumulate during the hypersensitive reaction (HR) of tobacco leaves infiltrated with an incompatible strain of the bacterial pathogen *Pseudomonas solanacearum* have been subdivided by sequence homologies into 6 families. Studies on the accumulation of the mRNAs encoded by these genes in compatible and incompatible plant-bacterial interactions have been carried out and indicate that the 6 cDNA clones can be subdivided into 2 groups. In one group corresponding to 3 cDNA clones, the maximal level of mRNA accumulation is similar in both types of interaction, whereas in the other group, maximal mRNA accumulation in leaves undergoing an HR is 3- to 7-fold higher than in leaves infiltrated with the compatible strain. Within each group, the timing and kinetics of accumulation of the corresponding mRNAs differ for each individual cDNA clone. Run-on experiments indicate that transcriptional activation of these genes plays a major role in the control of their expression. Genomic hybridizations have been performed and indicate that the mRNAs corresponding to the cDNA clones are encoded by multigene families (6 to 20 genes).

Introduction

The hypersensitive response (HR) is a very common plant reaction to avirulent and non-host pathogens and is almost always found associated with resistance [17] although its precise role is not at all clear [15]. This reaction is characterized by local cell death at the site of infection preventing further spread of the invading microorganisms. The development of the HR requires both physical contact between the plant and the pathogen, at least in the case of bacteria [31], and *de novo* synthesis of proteins in the two protagonists [6, 17, 30]. The synthesis of some non-constitutive components essential to the initial interaction between the plant and the pathogen, and the biochemical events leading to plant cell death may explain the absolute requirement for protein synthesis [25]. Whilst the molecular mechanisms leading to the HR are not known, several physiological changes including callose formation, synthesis of phenylpropanoids, vascular blockage, electrolyte leakage, lignification and changes in respiration rates have been demonstrated to be

associated with the cellular collapse and death of the plant cells undergoing this reaction. Moreover, particular gene products involved in the plant defence response such as enzymes of the phenylpropanoid pathway [9, 14, 19], enzymes possessing hydrolytic activities [1, 27], cell-wall proteins such as hydroxyproline-rich glycoproteins [22, 29], the pathogenesis-related (PR) proteins [16, 18, 20, 34] have been studied both *in vivo* in plants interacting with pathogens and *in vitro* in cell culture systems treated with biotic and abiotic elicitors of the host defence system.

In the present paper, the reaction of tobacco (*Nicotiana tabacum*) undergoing an HR after infiltration of leaves with an incompatible isolate of a phytopathogenic bacterium, *Pseudomonas solanacearum*, is reported. This pathogen, the causative agent of bacterial wilt, infects mainly solanaceous plants and has been classified into various races according to its host-range [4]. Moreover, genes involved in its pathogenicity have been extensively studied and mutants have been characterized which are affected in their ability to induce either the HR or the disease itself [2, 3]. Preliminary experiments using the tobacco-*P. solanacearum* model system have shown that the HR-inducing isolate, GMI1000, has an early and dramatic effect on plant gene expression. Significant changes in the mRNA population are not detected at this stage either with a HR^- isolate, GMI1178 or with a compatible isolate, K60 [26]. Here, we describe the isolation of several cDNA clones corresponding to plant mRNAs whose expression has been studied in plants challenged with compatible and incompatible bacterial isolates. Based on these results, 6 families of cDNA clones can be divided into 2 groups which differ primarily in the levels of expression of their corresponding mRNAs during the compatible versus the incompatible reaction.

Materials and methods

Plant material

Seedlings of *Nicotiana tabacum* cv. Bottom Special (gift from Dr L. Sequeira), of *N. sylvestris* and *N. tomentosiformis* were grown for 6 weeks in a greenhouse and then transferred for 3 weeks to a growth chamber. The youngest fully expanded leaves were excised and infiltrated *in vacuo* with the bacterial suspension as described previously [26]. After 2 to 4 hours, leaves loose their water-soaked appearance and show no sign of damage. In the incompatible interaction, the first signs of cellular death appear around 16 hours after infiltration. Eight hours later, the entire leaf is necrotic. At this time, no apparent damage is observed in leaves infiltrated with the K60 or the GMI1178 isolates. In the compatible interaction, the whole leaf becomes wilted between 48 to 60 hours after inoculation.

Bacterial strains and growth conditions

All the *P. solanacearum* strains used in this study were kindly provided by C. Boucher. The GMI1000 and K60 isolates [23] are wild-type *Pseudomonas solanacearum* races, virulent on tobacco, and on both tomato and tobacco, respectively. A hypersensitive reaction is induced within 24 hours by infiltration of the GMI1000 strain in tobacco leaves. The GMI1178 is a typical acridine orange resistant mutant obtained after growth of GMI1000 in the presence of 200 μg/ml of acridine orange [23].

P. solanacearum strains were grown at 30 °C in BGT medium [2]. Strains are conserved at 4 °C in one-quarter strength M63 [21]. GMI1178 is auxotrophic for methionine and the growth medium was therefore supplemented with L-methionine (16 μg/ml).

RNA extraction and isolation of poly(A)$^+$

Usually, at least 3 tobacco leaves from different plants were used for the RNA purifications. The RNA was extracted according to the method previously described [26]. Poly(A)$^+$ RNA was purified by 2 passages over oligo(dT) cellulose columns [28].

Construction of the cDNA library

The cDNA library was constructed from poly(A)$^+$ RNA purified from tobacco leaves inoculated with the GMI1000 *P. solanacearum*

isolate, 6 hours after infiltration and inserted into the *Pst* I site of plasmid pBR322 by the homopolymeric poly(dG · dC) tailing method [13, 24].

Differential screening of the cDNA library

[^{32}P]-labelled cDNAs were synthesized from mRNAs isolated from leaves infiltrated with the GMI1000 or the GMI1178 isolates according to the method of Darlix [7]. These 2 probes were then hybridized to the DNAs of transformed bacteria immobilized on Biodyne filters according to the recommendations of the manufacturer (Pall Corporation). After washing and autoradiography exposure, plasmid DNA was purified from the bacterial colonies which showed a stronger hybridization signal with the [^{32}P]-labelled probe synthesized from leaves undergoing an HR. Insert DNA from these plasmids were then [^{32}P]-labelled and hybridized with equivalent amounts of total RNA purified from leaves undergoing an HR or infiltrated with the GMI1178 strain by the dot blot method. After the second screening, the [^{32}P]-labelled inserts of the positive cDNA clones were finally hybridized to northern blots containing similar amounts (20 μg) of total RNA from leaves infiltrated with the GMI1000 or the GMI1178 strains. The size of the corresponding RNAs was then determined.

RNA gel electrophoresis and transfer

Total or poly(A)$^+$ RNA was electrophoresed on 1 to 1.5% agarose gel containing methylmercuric hydroxide [21].

RNA was transferred onto GeneScreen membranes following the protocol provided by the manufacturer (New England Nuclear). Total RNA was spotted on GeneScreen membranes with a BRL hybri-dot apparatus. Similar amounts of RNA (10 μg) were heated for 3 min at 90 °C, and then cooled on ice. The NaCl concentration was adjusted to 3 M final before filtering the samples.

Plasmid DNA preparation, purification and [^{32}P]-labelling of the cDNA inserts

Plasmid DNAs were purified minipreparations or large preparations by the alkaline lysis method [21]. The plasmid DNAs were digested with *Pst* I and the inserts separated from the vector pBR322 on a 1.5% agarose gel. They were then excised and purified by electroelution [5].

Inserts were [^{32}P]-labelled by the oligonucleotide labelling technique [10, 11].

Nuclei purification and run-on experiments

Nuclei were purified according to the method of Stout *et al.* [32]. The labelling of transcripts was done according to the protocol described by Willmitzer *et al.* [37]. About 10^7 cpm were used for each hybridization experiment.

RNA and DNA hybridizations

The hybridizations and washings of GeneScreen filters containing RNA or DNA were carried out according to the manufacturer's instructions (New England Nuclear).

Tobacco DNA extraction and genomic blot hybridization

Nuclear DNA was purified from *Nicotiana sylvestris*, *Nicotiana tomentosiformis* and *Nicotiana tabacum* cv. Bottom Special according to the protocol described by Dellaporta et al. [8]. Following electrophoresis on 1% agarose gels, 20 μg of DNA digested with various restriction enzymes was transferred onto Biodyne membranes (Pall corporation) and hybridized to the [^{32}P]-labelled purified inserts according to the method of Wahl *et al.* [35]. Hybridizations and washings were done at 37 °C following the same protocol.

Results

Isolation of cDNA clones corresponding to mRNAs with enhanced levels during the hypersensitive response

A cDNA library was constructed from mRNAs isolated from leaves 6 hours after infiltration with the incompatible *Pseudomonas solanacearum* strain, GMI1000. Eleven thousand clones were obtained and a differential screening was performed using [^{32}P]-labelled cDNAs prepared

Table 1. cDNA clones isolated.

Clone	Insert size	mRNA corresponding size
201	600 bp	750 b
203	600 bp	1200 b
239	1000 bp	1200 b
235	1200 bp	1500 b
246	750 bp	800 b
208	350 bp	800 b
332	350 bp	800 b
202	300 bp	800 b
232	250 bp	800 b
319	800 bp	1600 b
328	750 bp	1600 b
514	400 bp	1600 b
515	900 bp	1600 b
233	550 bp	1600 b

from mRNAs of leaves infiltrated either with the wild-type HR-inducing isolate GMI1000, or with the avirulent, non-HR-inducing derivative GMI1178. Both strains are isogenic and the latter strain differs only by a deletion of some *hrp* genes carried by a megaplasmid [3]. Fourteen cDNA clones corresponding to genes whose expression is greatly enhanced at this stage of development of the HR were thus isolated. The size of the corresponding mRNAs was determined by northern analysis with poly(A)$^+$ RNA purified from leaves inoculated with the GMI1000 isolate. Experiments of cross-hybridization between the different inserts indicate that these clones correspond to 6 different genes (or gene families). These results are summarized in Table 1.

Two groups of genes which are expressed differently during the incompatible interaction

The kinetics of accumulation of the mRNAs corresponding to the 6 categories of cDNA clones (201, 203, 235, 246, 319 and 515) have been determined during the course of various types of plant-pathogen interaction. Tobacco leaves were infiltrated with the K60 (compatible), the GMI1000 (HR$^+$), the GMI1178 (HR$^-$) isolates, and finally with water. Total RNA was extracted at varied

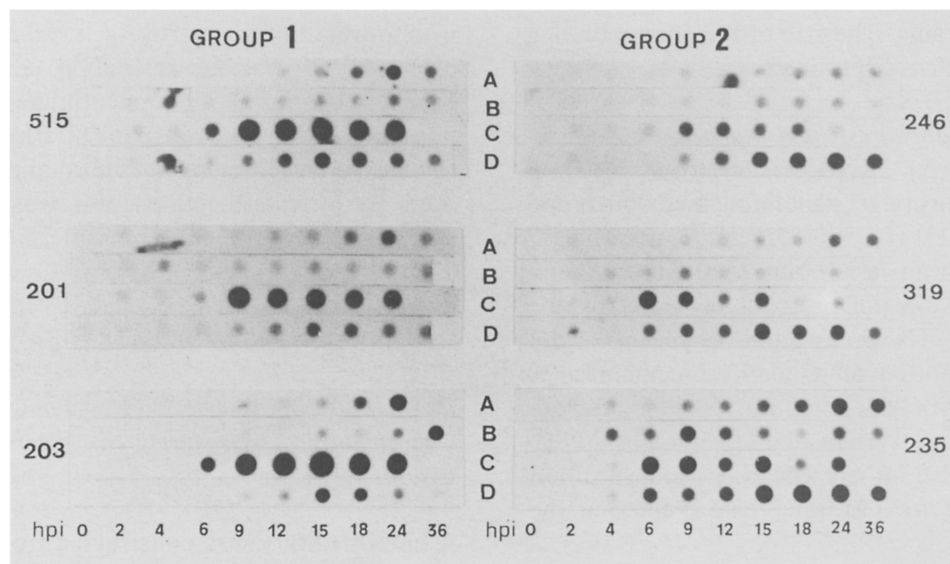

Fig. 1. Dot blot analysis of the expression of the mRNAs corresponding to the cDNA clones. Total RNAs were purified after various times of incubation, from leaves infiltrated with water (A), the GMI1178 (B), GMI1000 (C) and K60 (D) isolates and 10 μg spotted onto a GeneScreen membrane. They were then hybridized with the [^{32}P]-labelled inserts of the cDNA clones, washed and exposed as described in Materials and methods. In the case of the HR (C), it was not possible to take a sample at 36 hours because the plant cells are dead at this stage. hpi: hours post-inoculation.

times after inoculation and hybridized with the [^{32}P]-labelled inserts of the cDNA clones after filter transfer. The results (Fig. 1) indicate that the 6 families of cDNA clones can be subdivided into 2 groups, the main difference between them being the level of expression of their mRNAs during the compatible and the incompatible reaction.

In the first group, consisting of cDNA clones 201, 203 and 515 (Fig. 1, left lanes), mRNA accumulation starts 6 hours after inoculation with the GMI1000 isolate (between 6 and 9 hours for the clone 201) (row C). It is maximal between 9 and 15 hours and then decreases only very slightly between 15 and 24 hours after inoculation (Fig. 1, left panels). In the compatible reaction (row D), RNA transcripts appear later (between 9 and 12 hours), reach a maximal level around 15 hours and decrease significantly in the later stages of infection. The ratio between the maximal level of mRNA accumulation in the incompatible versus the compatible reaction is between 3 and 7 in this group of cDNA clones. In contrast with the second group of cDNA clones (Fig. 1, right panels), the mRNAs reach approximately the same levels in both types of interaction. In leaves inoculated with the GMI1000 isolate (row C), a transient increase in the amount of mRNA is observed between 6 and 12 hours. In the compatible reaction (row D), the mRNA accumulation is slightly delayed in the case of cDNA clone 246 starting between 12 and 15 hours, reaching a maximum between 18 and 24 hours after inoculation and then remaining at a high level. For the cDNA clones 235 and 319, mRNA accumulation starts earlier (around 6 hours), reaches a plateau and then decreases after 24 hours (clone 319) or increases until 15 hours and then remains almost constant (clone 235). It must be stressed that experiments with different batches of tobacco plants grown under the same conditions give slightly different results with regard to the timing and extent of mRNA accumulation. However, in all of our studies, the 2 groups of cDNA clones have always been clearly distinct in terms of the difference in the levels of mRNA accumulation in the compatible versus the incompatible reaction. The amount

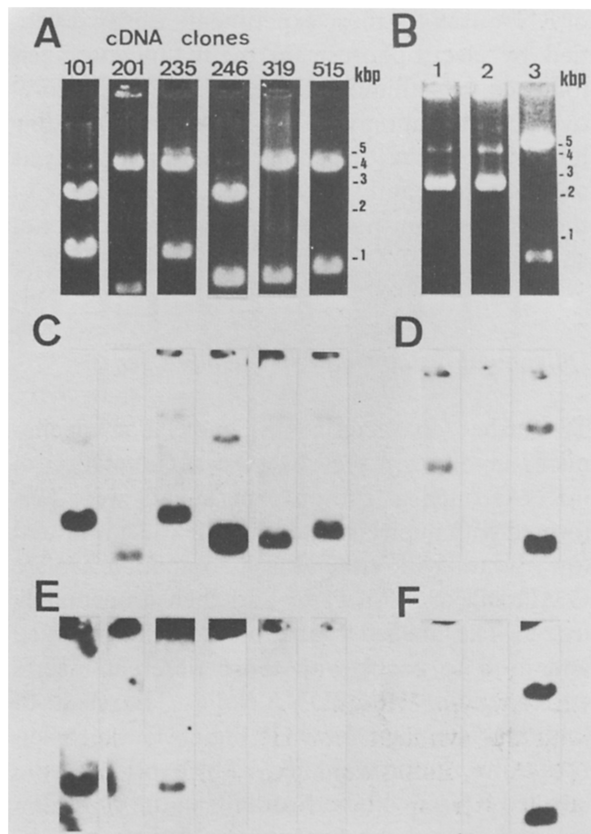

Fig. 2. Transcription of the genes corresponding to the cDNA clones, in isolated nuclei from leaves undergoing an HR and from control leaves. The plasmid DNAs containing the various inserts (pUC 19 for the cDNA clones 101 and 246, pBR322 for the cDNA clones 201, 235, 319 and 515) were digested with *Pst* I, electrophoresed on a 1.2% agarose gel, stained with ethidium bromide (Panel A). The insert of clone 203 was subcloned in M13 in both orientations. Both single-stranded DNAs were electrophoresed and, after ethidium bromide staining (Panel B, lanes 1 and 2), transferred onto a GeneScreen membrane. Nuclei were isolated from leaves infiltrated with GMI1000 and incubated for 9 hours, and from leaves infiltrated with water and frozen immediately. The transcripts were [^{32}P]-labelled, purified and hybridized with the digested plasmid DNAs as described in Materials and methods. Panels C and D: Autoradiograms obtained after hybridizing RNAs transcribed within the nuclei of leaves undergoing an HR. Panels E and F: Autoradiograms obtained after hybridizing RNAs transcribed from control leaves. The ubiquitin encoding cDNA clone, pHUB14-38, was digested with *Xho* I (Panel B, lane 3). Molecular weight markers are indicated on the right-hand side of panels A and B.

of RNA used for these experiments was first verified by electrophoresis in methyl-mercury gels followed by ethidium bromide staining. Moreover, hybridization with a cDNA clone encoding human ubiquitin [36] showed that mRNA levels are not significantly affected during the course of the various plant-pathogen interactions (data not shown).

Differential regulation of HR-induced genes

To further characterize the molecular mechanisms involved in the observed accumulation of mRNAs, nuclear run-on experiments were performed with nuclei isolated from leaves infiltrated for 9 hours with either the incompatible isolate GMI1000, or with water and then immediately frozen. The labelled transcripts were then purified and used to probe with the 6 different inserts corresponding to the cDNA clones. The results of this experiment are shown in Fig. 2. The specificity of transcription was checked by hybridizing the labelled RNAs with both strands of the clone 203 inserted in the bacteriophage M13 (panels B, D, F lanes 1 and 2). Only one strand of clone 203 hybridizes to the labelled RNA (lane 1). Controls including a ubiquitin cDNA clone [36] (panels B, D, F lane 3) and a clone isolated from our cDNA library and whose steady-state levels of mRNA are not significantly affected during the HR (clone 101) exhibited the same level of hybridization with the labelled transcripts purified from both control (panel E) and inoculated leaves (panel C). The clones 201, 246, 319 and 515 hybridize only with labelled mRNAs of inoculated leaves (panel C), indicating that transcriptional activation of the genes corresponding to these cDNA clones is the major cause of the observed mRNA accumulation. However, hybridization of the clone 235 was also detected in the control (panel E) although to a lower extent than in leaves undergoing the HR. This suggests that this gene may have a more complex type of regulation.

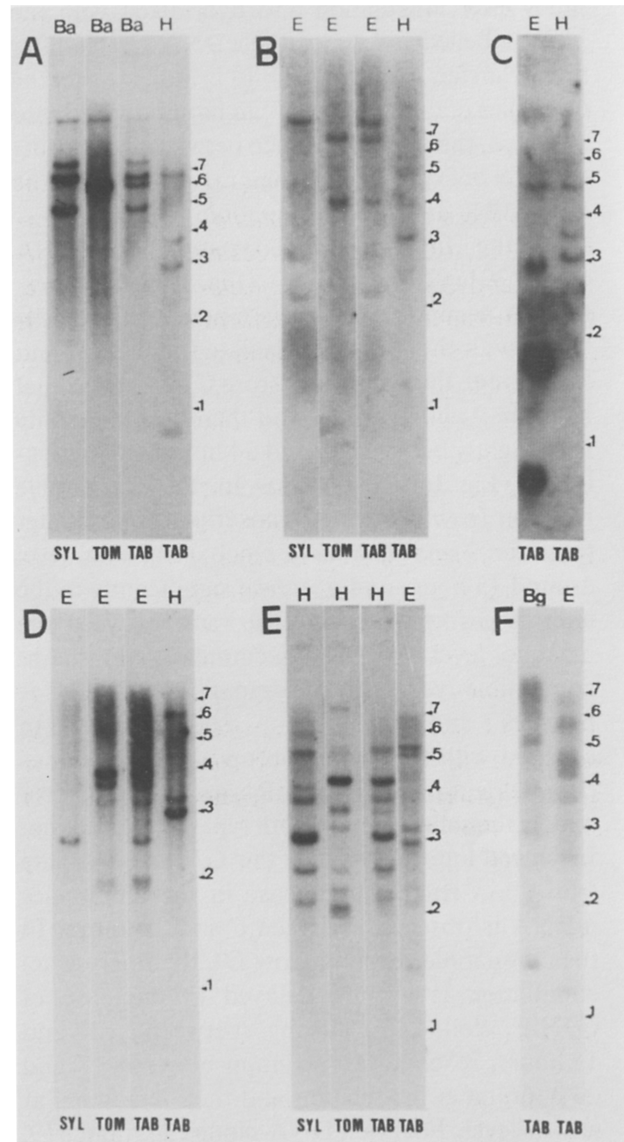

Fig. 3. Southern blot analysis of the tobacco genes corresponding to the 6 different cDNA clones. Nuclear DNAs purified from *N. sylvestris* (lanes SYL, panels A, B, D, E), from *N. tomentosiformis* (lanes TOM, panels A, B, D, E) and from *N. tabacum* (lanes TAB, panels A, B, C, D, E, F) were restricted with various restriction enzymes: E = *Eco* RI; Bg = *Bgl* II; H = *Hin*d III; Ba = *Bam* HI. After electrophoresis and transfer, the purified inserts of cDNA clones 203 = panel A, 515 = panel B, 201 = panel C, 246 = panel D, 319 = panel E, 235 = panel F, were used as hybridization probes. Size markers are indicated to the right of each panel, in kb.

The mRNAs corresponding to the cDNA clones are encoded by small multigene families

In order to discard the possibility that the mRNAs corresponding to the cDNA clones are encoded by bacterial genes, genomic hybridizations were realized. *Nicotiana tabacum* is amphidiploid, obtained by crossing *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. Therefore, the experiments were performed using both parental species and *Nicotiana tabacum*. The results shown in Fig. 3 indicate that the copy number of genes corresponding to cDNA clones 246, 203, 201 and 515 is low (around 4 to 6 copies). It appears to be higher for cDNA clones 235 and 319. One interesting point is that the number of bands corresponding to some cDNA clones is different in the 2 parental species. For instance, a single major band hybridizes to cDNA clone 246 in *Nicotiana sylvestris* whereas 3 bands are present in *Nicotiana tomentosiformis* (Fig. 3, panel D, lanes SYL and TOM). The pattern of hybridization of cDNA clone 201 with *Nicotiana tabacum* DNA digested with *Eco* RI (panel C, lane TAB) shows the presence of 2 strong signals (M_r = 700 bp and 1700 bp). These 2 bands are caused by contaminating vector DNA in the probe.

Discussion

In this study, 14 cDNA clones were isolated by differential screening of a cDNA library prepared from mRNAs isolated from tobacco leaves, 6 hours after infiltration with an incompatible isolate of *P. solanacearum*. These clones, corresponding to genes whose expression is greatly enhanced in the HR, can be regrouped into 6 families according to the size of the corresponding mRNAs and cross-hybridization experiments. Restriction maps of the various inserts have been established and show differences in the nucleotide sequences of cDNA clones belonging to the same family (for instance, cDNA clones 203 and 239 or cDNA clones 319 and 328, data not shown). This suggests that the corresponding mRNAs are encoded by several closely related genes. These observations have been confirmed by genomic hybridizations: indeed, cDNA clones 246, 203, 201 and 515 are represented by 4 to 6 copies whereas larger multigene families (10 to 20 copies) encode mRNAs corresponding to cDNA clones 319 and 235. In addition, the 2 parental strains, *N. sylvestris* and *N. tomentosiformis*, do not appear to contribute equally to the number of genes found in *N. tabacum*.

In several studies, the accumulation of pathogenesis-related proteins has been correlated with HR in tobacco and other plant species [34]. In order to ask if some of the cDNAs that we have isolated correspond to such proteins, total RNA from leaves sprayed with salicylic acid, a common inducer of PR proteins [33], was hybridized with the various [^{32}P]-labelled inserts of the cDNA clones. No mRNA accumulation was observed under these conditions (data not shown). Since some PR proteins have been identified as β-1,3-glucanases [18], hybridization was also carried out with a β-1,3-glucanase cDNA clone [12] and in this case, the corresponding mRNA accumulates as expected (data not shown). These results strongly suggest that these six families of cDNA clones do not encode salicylic acid-inducible PR proteins.

The study of the kinetics of expression of the mRNAs corresponding to the various cDNA clones indicates that they can be classified into 2 groups. Indeed, the clones 201, 203 and 515 correspond to mRNAs which might be called HR-related in the sense that their maximum levels of expression in the incompatible reaction are about 3- to 7-fold higher than in the compatible reaction. On the other hand, the mRNAs corresponding to clones 246, 235 and 319 accumulate to a similar level in both types of interaction. However, each individual clone has a very specific pattern of expression in the 2 types of plant-pathogen interaction.

The inoculation method used in this study which consists in leaf detachment and the infiltration of bacteria *in vacuo* into tobacco leaves is a quite stressful procedure. However, this treatment does not significantly affect the expression

of the genes corresponding to the cDNA clones that we have studied although, in the later stages of infection, a weak accumulation of the mRNAs is observed in control leaves infiltrated with water and the GMI1178 isolate (Fig. 2, rows A and B). This would suggest that their gene products are not related to some generalized stress and to wounding. Moreover, the observation that the mRNA accumulation starts early (approximatively 6 hours after inoculation) and is maximal long before any apparent symptom is visible, supports the idea that gene activation does not simply result from generalized cell collapse. These observations do not apply to cDNA clone 235 whose transcripts accumulate early in the control leaves. Using the same tobacco-*P. solanacearum* system, we have been able to show that the kinetics of expression of mRNAs corresponding to either a tobacco β-1,3-glucanase cDNA clone or a chitinase cDNA clone are very similar in leaves infiltrated either with water or with the various bacterial isolates used in this study [12]. These results explain why no PR protein encoding cDNA was isolated by our screening method and suggest that these transcripts accumulate in response to the stress produced by our technique of inoculation. However, several studies indicate that they do participate in the physiological events which prevent the spread of the pathogen.

We have no direct evidence that the gene products corresponding to the cDNA clones of group 1 described in this study are directly involved in the establishment of the HR. These genes may be activated by some physiological changes which take place in the cascade of events leading to cell collapse. They may also correspond to enzymes involved in the production of toxic compounds which may eventually provoke cell death. The kinetics of expression of mRNAs corresponding to the cDNA clones of group 2 resemble more closely those of the so-called 'defence' genes already described. The nucleotide sequences of the cDNA clones have been determined (data not shown) and do not correspond to known genes.

Finally, transcriptional control plays a major role in the regulation of expression of the genes corresponding to these cDNA clones. Clone 235 is, however, a notable exception since transcripts can be found in the control leaves in which no mRNA can be detected. In this case, one must assume that post-transcriptional events regulate the expression of this gene.

The 'HR-related' cDNA clones are particularly interesting since, to our knowledge, such genes have not yet been described. They may provide useful and specific tools for the study of a factor(s) involved in the early steps leading to the HR. Therefore, genomic clones corresponding to some of the cDNA clones described in this study have been isolated and are being characterized. Moreover, the influence of various factors such as phytohormones and elicitors on their expression is being studied and should give useful information concerning the physiological changes which take place during the incompatible interaction.

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