Local and Systemic Induction of Two Defense-Related Subtilisin-Like Protease Promoters in Transgenic Arabidopsis Plants. Luciferin Induction of *PR* Gene Expression[1]

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Following a pathogenic attack, plants are able to mount a defense response with the coordinated activation of a battery of defense-related genes. In this study we have characterized the mode of expression of the *P69B* and *P69C* genes from tomato (*Lycopersicon esculentum* Mill.), which encodes two closely related subtilisin-like proteases associated with the defense response. We have compared the mode of gene regulation in heterologous transgenic Arabidopsis plants harboring promoter-β-glucuronidase (*GUS*) and promoter-luciferase (*LUC*) gene fusions for these two genes. These studies revealed that the *P69B* and *P69C* promoters are induced by salicylic acid as well as during the course of both a compatible and an incompatible interaction with *Pseudomonas syringae.* Furthermore, *P69B* and *P69C* expression takes place in both the local and the distal (noninoculated) leaves upon inoculation with bacteria but following different and unique tissue-specific patterns of expression that are also different to that described for most other classical *PR* genes. Also, we report that luciferin, the substrate for the reporter luciferase (*LUC*) gene, is able to activate expression of *PR* genes, and this may pose a problem when using this gene reporter system in studies related to plant defense.

Plants are equipped with an array of defense responses to prevent their invasion by pathogens. Some of these defensive tools are already established in the plant, whereas others are inducible upon perception of the pathogen. The most common feature of disease resistance in incompatible plant-pathogen interactions is the rapid development of a cell death process at the infection sites (the so-called hypersensitive response (HR) (Agrios, 1988). The onset of HR in turn activates a signaling process throughout the plant that makes it more refractory to subsequent infections by a broad spectrum of pathogens. This latter response is coined systemic acquired resistance (SAR) (Ross, 1961; Hunt and Ryals, 1996; Ryals et al., 1996).

Salicylic acid (SA) is a master regulatory molecule that accumulates to considerable levels following pathogen recognition, is implicated in the promotion of HR, and is also necessary for SAR induction (Yalpani et al., 1991; Shirasu et al., 1997; for review, see Hammond-Kosack and Jones, 1996;). An elevation in the endogenous level of SA or exogenous application of SA or its synthetic analogs results in a selective and concerted activation of a plethora of genes (SAR genes) encoding proteins related to defense (Ward et al., 1991; Lawton et al., 1993). A major subset of these proteins is known as pathogenesis-related (PR) proteins, which comprises several families of proteins (Cutt and Klessig, 1992). The initial expression of *PR* genes takes place in dying tissues that are in direct contact with the pathogen and are thus developing HR. Later on, expression of *PR* genes is induced in the distal tissues during the course of SAR induction. This expression profile along with the demonstrated antimicrobial activity of some PRs (Mauch et al., 1988; Broglie et al., 1991; Zhu et al., 1994) lead to the suggestion that PRs are at least partially responsible for maintaining the disease-resistant state of the plant (Lawton et al., 1993).

A novel and interesting *PR* gene subfamily is that encoding members of the subtilisin-like Ser proteinases (EC 3.4.21.14) that represent an ancient family of evolutionarily conserved proteins (Siezen and Leunissen, 1997). In tomato plants, recent sequence comparison revealed that the subtilisin-like genes fall into five distinct subfamilies (Meichtry et al., 1999) with the P69 subfamily members being the best characterized so far. The P69 subtilisin-like proteases belong to a multigene family of high complexity that encodes protein isoforms of approximately 69 kD that accumulate extracellularly (Tornero et al., 1996, 1997a). Genomic clusters comprising a tandem array of four genes (*P69A*, *P69B*, *P69C*, and *P69D*) and a tandem array of two genes (*P69E* and *P69F*) encoding closely related P69 proteases were identified recently in tomato plants (Jordá et al., 1999, 2000). Detailed

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expression analysis of each of these genes revealed that they are tightly regulated by developmental and environmental cues in tomato plants (Jordá et al., 1999, 2000). The *P69A* gene was shown to be expressed constitutively, whereas the *P69D* gene showed transient expression in rapidly expanding leaves. At variance, the *P69E* gene is only expressed in root tissues, whereas the *P69F* gene is specifically expressed in hydathodes. Although *P69A*, *P69D*, *P69E*, and *P69F* gene expression is not induced over basal levels during pathogenesis, it cannot be ruled out that these genes are implicated in pathogenesis by acting as an early line of defense, as proposed for other constitutively expressed *PR* genes (Samac and Shah, 1991; Tornero et al., 1997b and references therein).

In marked contrast, two other gene members of the P69 family, namely *P69B* and *P69C*, do not show constitutive expression but are notably induced in tomato plants by infection with *Pseudomonas syringae* or upon treatment with SA (Jordá et al., 1999). This suggests that both, P69B and P69C, may play roles as active defense weapons against the attacking pathogens.

In the present paper we show a comparative study of the mode of gene regulation of the *P69B* and *P69C* genes in transgenic Arabidopsis plants harboring promoter-β-glucuronidase (*GUS*) and promoter-luciferase (*LUC*) gene fusions. Also, we show that luciferin, the substrate for the reporter luciferase (*LUC*) gene is able to activate expression of defenses in the plant, and this may pose a problem when using this reporter system in defense-related studies.

RESULTS

Local and Systemic Induction of Reporter Gene Expression Driven by *P69B* and *P69C* Promoters

The *P69B* and *P69C* genes were shown to be induced following infection of tomato plants with *P. syringae* (Jordá et al., 1999). To understand how this induction is regulated and where does it take place in the infected plant, we constructed different chimeric promoter fusions with the bacterial β-glucuronidase (*GUS*) and the firefly luciferase (*LUC*) reporter genes. These were introduction into Arabidopsis plants and used to study the transcriptional regulation of these genes by histochemical analysis of *GUS* activity or by capturing the luminescence emission derived from *LUC* expression in intact plants.

The chimeric *P69B::GUS* gene was constructed by fusion of the 2.6-kb region of the *P69B* 5′-promoter region (Jordá et al., 1999) to the ATG initiation codon of the *GUS* coding sequence and the 3′-untranslated region of the nopaline synthase (*NOS*) gene (Fig. 1) in the binary vector pBI101 (Jefferson et al., 1987). The chimeric *P69C::GUS* gene was constructed similarly by fusion of the 2.5-kb region of the *P69C* 5′ promoter (Jordá et al., 1999) to the ATG initiation codon of the *GUS* coding sequence in pBI101.

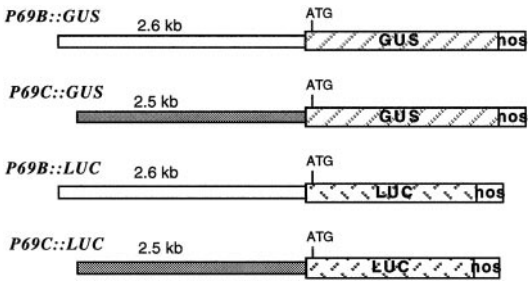

Figure 1. Schematic representation of the different *P69::GUS* and *P69::LUC* gene fusions. The diagonally striped boxes represent the *GUS* or *LUC* genes. The white box at the right represents the 3′-region of the nopaline synthase gene. The length of each of the promoter regions is shown above each construct in kilobase pairs. The ATG codon represents the first translation initiation codon that resides in the reporter gene.

Preliminary studies for the spatial distribution of *GUS* activity driven by these two *P69* promoters during different developmental stages of growth revealed no constitutive expression for either two genes (data not shown). To study the mode of gene induction following pathogen infection, we inoculated leaves of transgenic Arabidopsis plants with the bacterial pathogen *P. syringae* DC3000, carrying or not carrying the avirulence gene *Rpm1,* and the extent of *GUS* induction determined directly in leaf tissues by histochemical staining with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-gluc). These studies revealed that both *P69B* and *P69C* promoters are induced in both the local inoculated leaves as well as in distal (noninoculated) leaves from the same plant (Fig. 2). *P69B::GUS* expression follows a similar tissue pattern of induction upon inoculation with either the virulent or the avirulent bacteria. For both types of bacteria, the induced expression of *GUS* activity takes place in the inoculated leaves as well as in distal (noninoculated) leaves of the same plant (Fig. 2, A–E). This induction is always delimited to primary and secondary veins of the afflicted plant and with no preferential expression around the tissue zone showing the characteristic HR response (marked with arrow in Fig. 2D) when the plant was inoculated with the incompatible bacteria. Likewise, *P69C::GUS* expression was also induced by both bacterial strains and also was activated in local as well as in distal leaves (Fig. 2, F–J). However, at variance with the *P69B* promoter, the *P69C* promoter is activated with different tissue specificity. Its expression takes place in the form of spots scattered all along the leaf lamina. In the case of the incompatible interaction, neither of these promoters showed preferential expression in the region encompassing the HR lesion (marked with arrow in Fig. 2I).

Analysis of *P69B* and *P69C* Gene Expression in Transgenic Intact Plants

To study the expression pattern of the *P69B* and *P69C* genes in the entire plant in a non-destructive

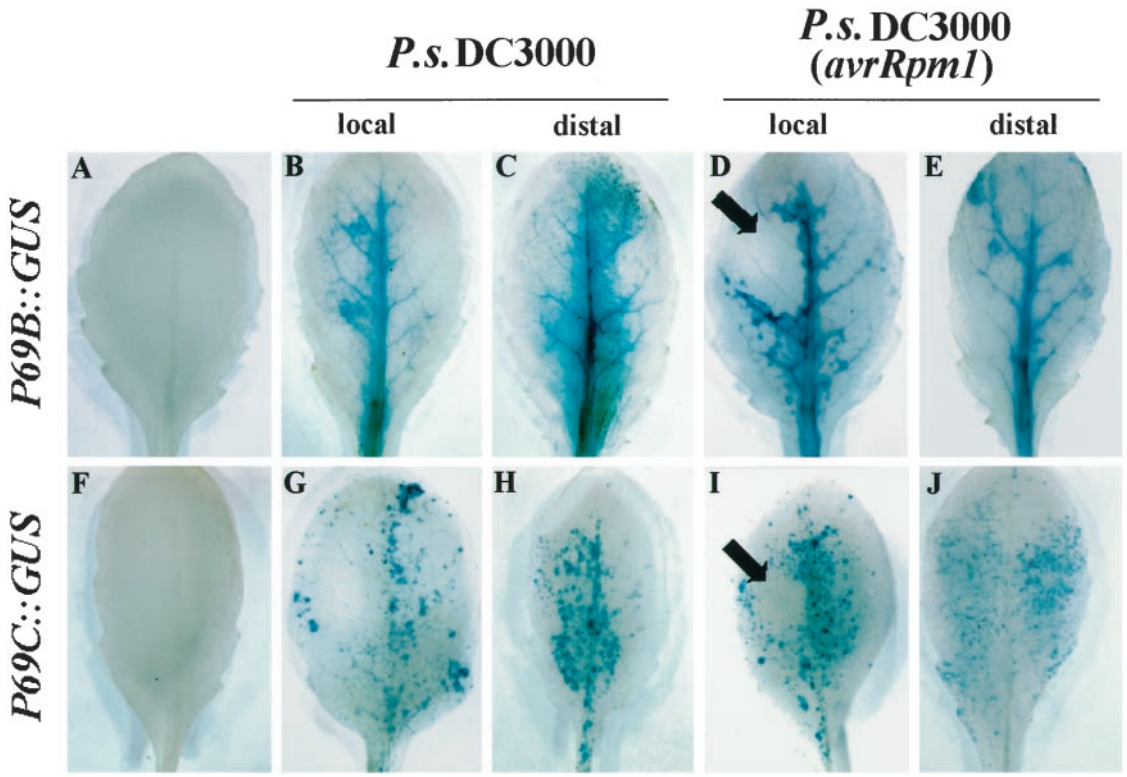

Figure 2. *GUS* staining patterns in rosette leaves of transgenic Arabidopsis (Col-0) plants carrying the *P69B::GUS* and *P69C::GUS* transgenes. Top, *GUS* staining pattern in leaves from *P69B::GUS* transgenic plants. Bottom, *GUS* staining pattern in leaves from *P69C::GUS*. A and F, *GUS* staining pattern in leaves from noninoculated plants. B and G, *GUS* staining pattern in leaves inoculated with *P.s.* DC3000. C and H, *GUS* staining pattern in distal (noninoculated) leaves from plants inoculated with *P.s.* DC3000. D and I, *GUS* staining pattern in leaves inoculated with *P.s.* DC3000 carrying the avirulent *Rpm1* gene. E and J, *GUS* staining pattern in distal (noninoculated) leaves from plants inoculated with *P.s.* DC3000 carrying the avirulent *Rpm1* gene. Leaves were analyzed 72 h after inoculation. The characteristic HR responses elicited in the inoculated leaves with the incompatible bacteria are indicated with arrows. The experiments were repeated with plants from at least three different transgenic lines for each construct and in all cases render similar results.

fashion, new gene constructs were generated in which the *GUS* reporter gene was replaced by the luciferase (*LUC*) reporter gene to render *P69B::LUC* and *P69C::LUC* gene constructs (Fig. 1). These new constructs were used to generate new Arabidopsis transgenic plants. The extent of *LUC* gene expression driven by each of these two promoters was followed in intact plants by capturing the luminescence emission in a CDC camera upon spraying plants with luciferin, the substrate of the LUC protein. The comparison of luminescence images revealed that these transgenic plants showed induced expression of the reporter *LUC* gene at 72 h postinoculation with both types of bacteria and with nearly undetectable expression in noninoculated plants (Fig. 3). In the case of *P69B::LUC* plants, image analysis of luminescence showed a preferential emission in the central veins of the rosette leaves (Fig. 3). In the case of *P69C::LUC* plants, luminescence emission is less concentrated and extends along the entire plant with preferential emission in leaf blades and not around veins (Fig. 3). Each of the two patterns of *LUC* gene expression was reproducibly generated when the plants where inoculated with either the compatible *(P.s.* DC3000) or with the incompatible (*P.s.* DC3000 *avrRpm1*) bacteria (Fig. 3).

Thus, from these experiments it can be concluded that the *P69B* and *P69C* genes are induced locally and systemically but with different tissue specificity. In neither case is the expression delimited to the necrotic zone where the majority of the PR genes have been shown to be expressed (Tornero et al., 1997b and references therein).

Luciferin and SA Are Potent Inducers of *P69B* and *P69C* Gene Expression

In contrast to the assay of *GUS* activity, the advantage of the LUC assay is that it is not destructive and thus allows studies of gene expression to be performed in the same plant at different times or following different treatments. In attempts to perform this type of study with the *LUC* gene driven by the *P69B* or the *P69C* promoters, transgenic plant harboring *P69B::LUC* or *P69C::LUC* constructs were inoculated with either virulent or avirulent bacteria, and the

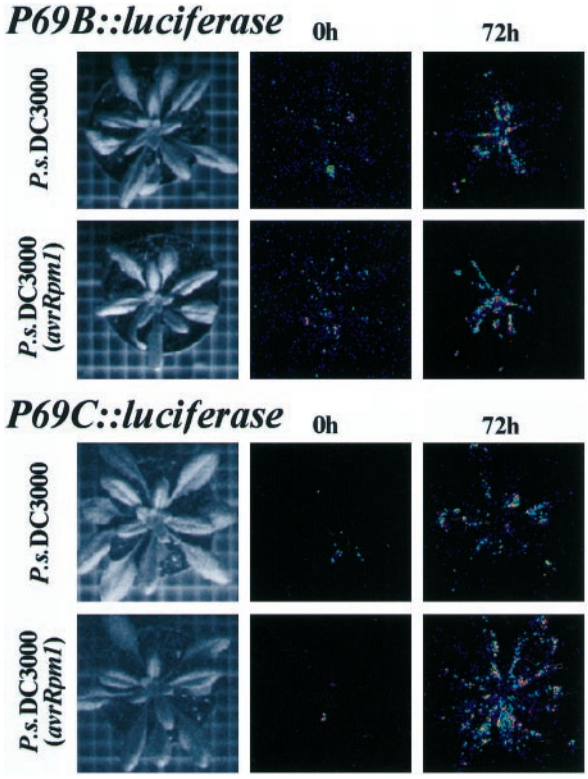

Figure 3. Monitoring of light emissions in entire *P69B::LUC* and *P69C::LUC* transgenic Arabidopsis plants by low-light video image analysis following inoculation with compatible and incompatible bacteria. Left, Standard photographs of the type of plants used in these experiments. Middle, Luminescence from plants at the time of inoculation (0 h) with either *P.s.* DC3000 or *P.s.* DC3000 (*avrRpm1*). Right, Luminescence from plants at 72-h postinoculation with either *P.s.* DC3000 or *P.s.* DC3000 (*avrRpm1*). At the times indicated, plants were sprayed once with 1 mM luciferin, and images were obtained immediately after 10 min of photon collection. The plants shown in each of the panels are different plants but derived from the same homozygous transgenic lines. The experiments were repeated with plants from at least three different transgenic lines for each construct and in all cases render similar results.

extent of luminescence emission due to *LUC* expression was recorded in the same plant at 24-h intervals. At each time point in the study the plant was sprayed with a 1-mM solution of luciferin to provide fresh substrate to detect newly induced *LUC* enzyme. This type of experiment, summarized in Figure 4, shows that for both gene constructs the activation of *LUC* expression following pathogen inoculation increased continuously along the time. However, a comparison of luminescence images among the many different experiments performed revealed that LUC induction was reproducibly higher than that observed previously (Fig. 3) in experiments in which LUC expression was determined in plants that received only a single treatment with luciferin. These dramatic differences in the extent of LUC induction suggested that presumably luciferin was acting as a potent inducer of *P69B* and *P69C* gene expression. To test this possibility, noninfected transgenic plants were sprayed every 24 h with a solution containing 1 mM luciferin, and the extent of LUC induction was recorded at 72 h. For comparison, other plants from the same transgenic lines were sprayed with a solution containing 0.5 mM SA, which is a potent inducer of defense-related genes. The experiment in Figure 5 shows that the sole application of luciferin is sufficient to bring activation of the *P69B* and *P69C* promoters in a manner similar to that provoked by an application of SA alone.

To verify if the real inducer of gene expression was luciferin or a side product of its ATP-dependent metabolic conversion (Karl and Holm-Hansen, 1976) in the living cells by the action of trace amounts of pre-existing *LUC*, the same experiment shown in Figure 5 was performed in transgenic plants containing *P69B::GUS* or *P69C::GUS*. Figure 6 shows that luciferin alone was able to induce *GUS* activity driven by the *P69B* and *P69C* promoters (Fig. 6, C and F), and these induction patterns were similar to those achieved upon treatment with SA alone (Fig. 6, B and E). It is interesting that both luciferin and SA activate the expression of these two genes in a tissue-specific pattern that coincides with that observed when expression was induced by bacterial infection. This suggests that these two molecules operate following a mechanism similar to that of the endogenous plant signal produced during pathogenesis.

To extend this observation to other defense-related genes, wild-type Arabidopsis plants were treated similarly with luciferin or SA, and total mRNA was extracted from these plants. Northern blots shown in Figure 7 demonstrate that *PR-1* and *PR-2* mRNAs accumulate in response to luciferin to an extent comparable with that achieved by SA. This observation thus reinforces the consideration that luciferin, as is true for SA, is a potent inducer of defenses in the plant. However, at this stage we cannot disregard the possibility that the true inducer of *PR* gene expression is a breakdown product of luciferine.

DISCUSSION

Here we describe the mode of expression of two closely related genes (*P69B* and *P69C*) from tomato plants encoding members of the PR family of P69 subtilisin-like proteases. Comparison of the *P69B* and *P69C* nucleotide and deduced amino acid sequences reveal remarkable similarity all along the open reading frames (87.3% identity) (Jordá et al., 1999). The observation that these two genes are tightly linked within an approximately 30-kb genomic cluster that comprise a total of four *P69*-like genes leads to the suggestion that this multigene family might have derived from a common ancestral gene by recent gene duplication events (Jordá et al., 2000). However, the high-sequence identity existing within the coding regions for these genes does not extend outside of the open reading frames, thus suggesting that the genes

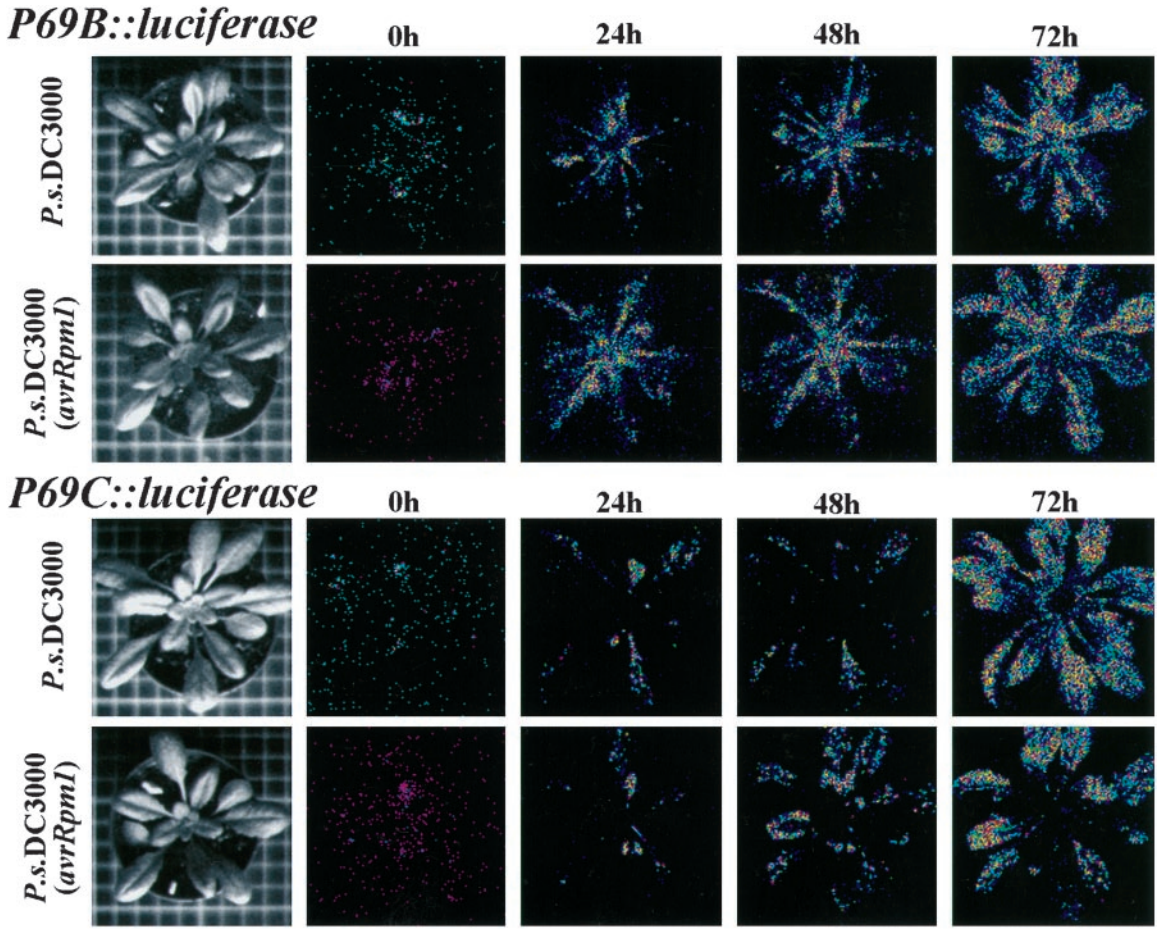

Figure 4. Monitoring of light emissions by low-light video image analysis in a single transgenic Arabidopsis plant during the course of a compatible or an incompatible interaction. Two leaves from each plant were inoculated with either *P.s.* DC3000 or *P.s.* DC3000 (*AvrRpm1*) and at each time point the plant was taken from the growth chamber, sprayed with 1 mM luciferin, and immediately the photon collection was performed for 10 min. This process was repeated in the same plant at 0, 24, 48, and 72 h postinoculation with the bacteria. The left column of pictures shows a standard photograph of the single plant used in each experiment. Top, Two plants derived from the same *P69B::LUC* transgenic line. Bottom, Two plants derived from the same *P69C::LUC* transgenic line. The experiments were repeated with plants from at least three different transgenic lines for each construct and in all cases render similar results.

may have acquired a different transcriptional regulation during evolution.

To understand how the *P69B* and *P69C* genes, previously shown to be up-regulated in diseased tomato plants, might be transcriptionally regulated during pathogenesis, transgenic Arabidopsis plants harboring different promoter-reporter gene constructs were generated. Analysis of transgenic plants containing the *GUS* or the *LUC* reporter genes under the control of the 5′-promoter region of the *P69B* or *P69C* genes did not reveal any detectable constitutive expression for any of them in healthy plants. Conversely, both promoters are able to drive expression of either *GUS* or *LUC* when transgenic plants are inoculated with the compatible *P.s.* DC3000 or the incompatible *P.s.* DC3000 *AvrRpm1*. This induced expression is observable in both the local inoculated leaves as well as in distal noninoculated leaves of the same plant. This type of gene expression pattern coincides with that reported for other defense-related genes (referred to as SAR genes, Ryals et al., 1996), which are concomitantly induced in inoculated, as well as in upper noninoculated leaves (Brederode et al., 1991; Ward et al., 1991; Lawton et al., 1993). However, when the infected transgenic plants are analyzed by histochemical staining to detect expression of *GUS* activity driven by the *P69* promoters, differences with the mode of expression of other inducible *PR* genes are observed. Our studies reveal that while the induced expression driven by the *P69B* promoter is restricted to the veins of the inoculated plant, the expression pattern derived from the *P69C* promoter takes place in group of cells that are distributed all along the leaf blade and not in the veins. Furthermore, both *P69B* and *P69C* gene expression takes place far away from the necrotic lesion that is derived from the HR during

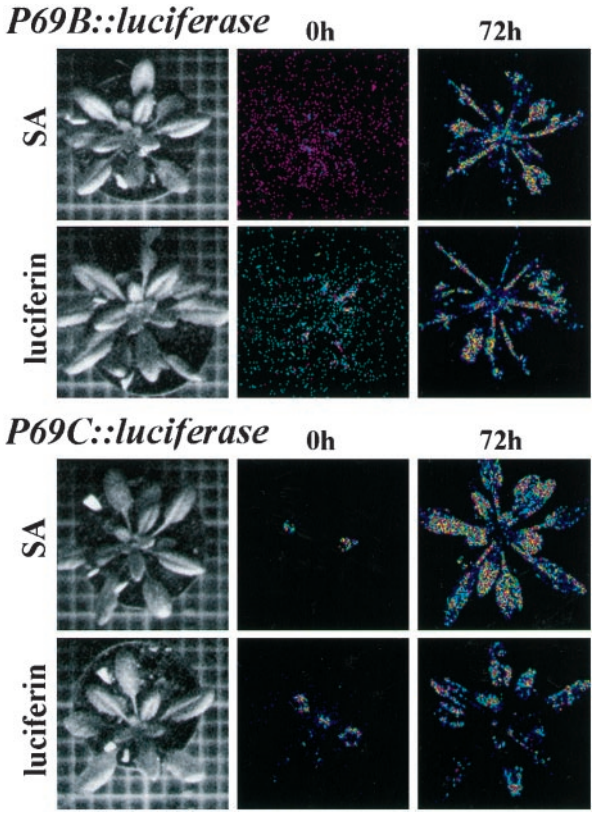

Figure 5. Effect of exogenous application of SA and luciferin on the activation of *P69B::LUC* and *P69C::LUC* gene expression. *P69B::LUC* transgenic Arabidopsis plants (top) and *P69C::LUC* transgenic Arabidopsis plants (bottom) were sprayed three times (at 24-h intervals) with a solution containing 0.5 mM SA or 1 mM luciferin. The same plants were monitored for light emissions by low-light video image at 0 or 72 h after receiving the first chemical treatment. Images were obtained after 10 min of photon collection.

the incompatible interaction. These two tissue patterns of expression are highly different to that observed for most classical *PR* genes, which are highly expressed in the tissues that surround the HR lesions (Ohshima et al., 1990; Van der Rhee et al., 1990; Eyal et al., 1993; Meller et al., 1993; Uknes et al., 1993; Alonso et al., 1995; Tornero et al., 1997). Thus the confined transcriptional activation observed for the *P69B* and *P69C* genes constitutes examples, to some extent unexpected, of novel and precise mechanisms of expression of *PR* genes.

Regulatory elements controlling pathogen-induction of *PR* genes have been studied in a number of cases; however they are widely different, making it difficult to define a minimal promoter necessary for pathogen induction of different *PR* genes. Lebel et al. (1998) recently have identified that the CGTCA motif (*as-1* element) is important for SA induction of *PR-1* gene expression. However, this 5-bp element is not found in any of the two *P69* promoters, and this may suggest that the regulation of the latter is in part different to that of *PR-1*. Also, the conserved GC-CGCCTC DNA motif is present in the promoter region of a number of genes that encode "basic" isoforms of PRs and appears to be necessary for induction of these genes around the HR zone in an ethylene-dependent manner (Eyal et al., 1993; Meller et al., 1993; Alonso et al., 1995; Tornero et al., 1997 and references therein). This cis-acting element is not found in any of the two promoters under consideration, and since neither of them respond to exogenous ethylene (data not shown), it is tempting to speculate that the observed induction of these two

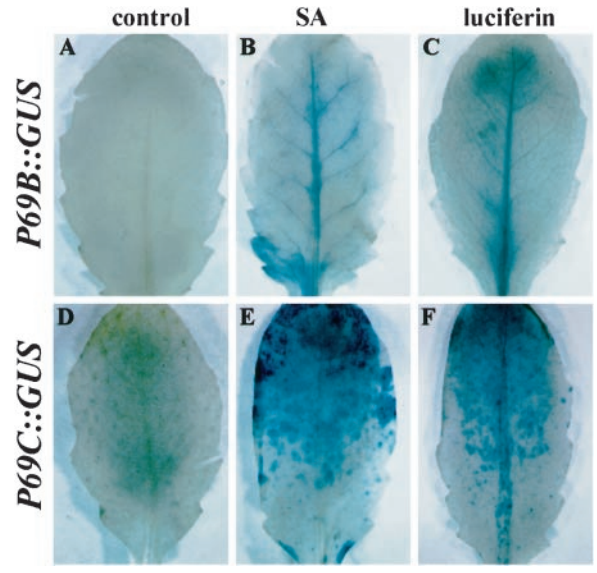

Figure 6. Effect of SA and luciferin on the activation of *P69B::GUS* and *P69C::GUS* gene expression. *P69B::GUS* transgenic Arabidopsis plants (top) and *P69C::GUS* transgenic Arabidopsis plants (bottom) were sprayed three times (at 24-h intervals) with a solution containing 0.5 mM SA or 1 mM luciferin, and *GUS* expression was detected by histochemical staining of leaves with X-gluc. A and D, *GUS* staining pattern before treatment. B and E, *GUS* staining pattern at 72 h after SA treatments. C and F, *GUS* staining pattern at 72 h after luciferin treatments.

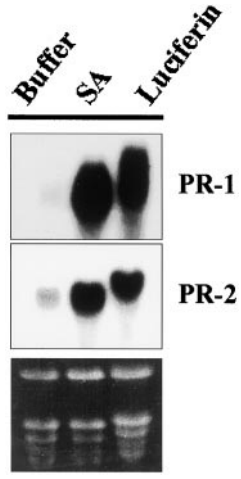

Figure 7. Northern-blot analyses of endogenous Arabidopsis *PR-1* and *PR-2* gene expression at 48 h following treatment of plants with SA and luciferin.

P69 genes is not controlled by the level of endogenous ethylene produced by infection. Thus this might explain why these two promoters avoid expression around the necrotic lesion during the HR.

SA is a master regulatory molecule that accumulates following pathogen recognition. It has been proposed that SA is a mobile signal that can be transported to distal tissues from the infection site to participate in SAR by activating defenses systemically (for review, see Hammond-Kosack and Jones, 1996). Also, when applied exogenously, SA induces expression of PR genes (Ohshima et al., 1990; Van der Rhee et al., 1990; Ward et al., 1991; Yalpani et al., 1991; Uknes et al., 1993). Since *P69B* and *P69C* are responsive to SA in a tissue-specific manner coincident with that achieved in infected plant, this favors the interpretation that SA may be the likely signal that set in motion the transcriptional activation of these two genes in such a precise manner.

How such a variety of different cell-type-specific expression patterns has evolved for a common set of pathogen-inducible *PR* genes and how are they coordinately activated during disease development either locally or in distant tissues in such a precise fashion remains enigmatic. The availability of such a variety of pathogen-inducible promoters, including the *P69B* and *P69C* presently described, will help increase our understanding of the complex biological signaling processes that are set in motion during disease resistance in plants. Also, they may be used as molecular tools in experiments aimed to engineer specific aspects of the resistance of plants to challenging pathogens.

Derived from the present study, additional considerations are the results from the observed transcriptional activation of *PR* genes when luciferin is used as a substrate to detect the activity of the reporter firefly luciferase (LUC) protein in vivo. Luciferin, a 6-hydroxy-benzothiazol (Karl and Holm-Hansen, 1976; Koncz et al., 1990), or one of its breakdown products, is presumably acting as an analog of SA thus priming the expression of SA-inducible genes in a manner similar to that of other benzoic acid derivatives (e.g. 2,6-dichloroisouicotinic acid). With this observation, we want to bring to the attention of researchers in the field of defense-related genes that the use of the *LUC*/luciferin system might bring false interpretation of results and that experiments using this gene reporter system (e.g. those directed toward isolation of mutant plants with altered expression patterns of defense-related genes) should be performed with care.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Treatments

Arabidopsis (Col-0) plants were grown at 22°C in growth chambers programmed for a 14-h light and 10-h dark cycle. Rosette leaves were either sprayed with SA (0.5 mM), luciferin (1 mM), or buffer alone (50 mM phosphate buffer, pH 7.2) as described before. Leaves were also inoculated with *Pseudomonas syringae* DC3000 carrying or not carrying the avirulence *Rpm1* gene as previously described (Jordá et al., 1999).

Promoter Constructs, Plant Transformation, and Analysis of Transgenic Plants

Oligonucleotides GEN69b (5′-GCCCGGGGGCTAGCTAATACAACAAGTG-3′) and GEN69c (5′-GCCCGGGGGCTGCAAATACAAGAAG-3′) in combination with the T7 oligonucleotide, served as primers for the incorporation of a synthetic *Sma*I restriction site in each promoter by site-directed mutagenesis (Kunkel et al., 1987). These primers introduced the *Sma*I site at positions −1 relative to the translation initiation sites in each gene. *Sma*I-*Bam*HI fragments encompassing 2.6 kb and 2.5 kb of the promoter regions of *P69B* and *p69C*, respectively, were cloned upstream of the *uidA* gene in *pBI101.1* (Jefferson et al., 1987) to generate plasmids *pP69E::GUS* and *pP69F::GUS*. For transcriptional fusions with the luciferase (*LUC*) gene, these constructs were digested with *Sma*I-*Sac*I to release the *GUS* coding region that was replaced by the *LUC* coding region. The resulting transcriptional fusions were verified by nucleotide sequence analysis using specific primers. The constructs were introduced into Arabidopsis (Col-O) by *Agrobacterium tumefaciens*-mediated transformation (Bechtold et al., 1993). Transformants were selected on Murashige and Skoog agar medium containing kanamycin, transferred to soil, and allowed to self pollinate. The transgenic lines were assayed for GUS activity by a fluorimetric assay or by an in situ assay using the chromogenic substrate X-gluc (Jefferson, 1987) and for luciferase activity as described below. For each construct, expression was determined in at least four independent transgenic lines.

LUC Imaging

Imaging with the firefly *LUC* reporter requires application of the exogenous substrate luciferin. Luciferin (Promega, Madison, WI) was dissolved in water and stored frozen as a 1-mM solution. This 1-mM solution was applied uniformly by spraying onto plants. For LUC imaging, the plants were kept for 2 min in the dark after the luciferin application. The imaging system consists of a high-performance CCD camera mounted in a dark chamber, a camera controller, and a computer. Image acquisition and processing were performed with the WinView software provided by the camera manufacturer (Hamamatsu, Japan). Exposure time was 10 min unless stated otherwise.

RNA-Blot Analysis

RNA was purified as described (Jordá et al., 1999), and 15 μg of total RNA were electrophoresed on 1% agarose gels containing formaldehyde and blotted onto Nytran membranes (Schleicher & Schull, Keene, NH). Equal loading of RNA was verified by ethidium bromide staining of

the gel before transfer to the membrane. Radiolabeled probes were prepared by random priming using T7 polymerase (Pharmacia Biotech, Piscataway, NJ). Hybridization and washing conditions of filters were done as described (Jordá et al., 1999).

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