Research Note

Class I and Class II *avr*D Alleles Direct the Production of Different Products in Gram-Negative Bacteria

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The *avr*D gene cloned from *Pseudomonas syringae* pv. *tomato* causes gram-negative bacteria to produce several unique secondary metabolic compounds. Two of these compounds, called syringolides, account for most of the *avr*D elicitor activity in soybean plants carrying the *Rpg*4 disease resistance gene (Keen and Buzzell 1991; Keen *et al.* 1990; Kobayashi *et al.* 1990a; Midland *et al.* 1993; Smith *et al.* 1993). A nonfunctional *avr*D allele was also cloned from *P. s.* pv. *glycinea* that was 86% identical to the *P. s.* pv. *tomato* allele (Kobayashi *et al.* 1990b) but directed little or no elicitor activity in gram-negative bacteria (Keen *et al.* 1990). Recently, three additional *avr*D alleles were characterized from two other *P. syringae* pathovars (Yucel *et al.* 1994; Yucel and Keen 1994). One of these genes (*P. s.* pv. *lachrymans* allele 1) was highly homologous to the *P. s.* pv. *tomato avr*D allele, and these were accordingly called class I alleles. The class II *avr*D alleles from *P. s.* pvs. *glycinea* and *phaseolicola* and *P. s.* pv. *lachrymans* allele 2 were highly homologous to each other but less homologous to the class I alleles. In this paper, we report that the two different classes of *avr*D alleles are also functionally unique, since they direct the synthesis in *Escherichia coli* and *P. s.* pv. *glycinea* of different elicitors of the soybean hypersensitive response.

E. coli DH5α cells carrying expression plasmids with various *avr*D alleles (Yucel and Keen 1994) were grown on 500 ml of M9 glucose (0.2%) medium supplemented with thiamine (4 μg/ml), ampicillin (75 μg/ml), and 0.4 mM isopropyl thiogalactoside. *P. s.* pv. *glycinea* race 4 cells carrying certain of the *avr*D alleles cloned in pDSK519 (Keen *et al.* 1988) were grown on 1 L of M9 glucose (0.4%) medium supplemented with 0.1% casamino acids and kanamycin at 50 μg/ml. All cultures were shaken at 28° C for approximately 16 hr. The cell-free culture fluids were adjusted to approximately pH 5.5 with HCl and successively extracted twice with ethyl acetate (Keen *et al.* 1990). Following removal of water from the combined organic phases with solid $MgSO_4$, they were taken to dryness at 50° C and partially purified on normal-phase Sep-Pak cartridge (Waters Associates, Milford, MA) as described by Midland *et al.* (1993). The 60% ethyl acetate in $CHCl_3$ fraction was then dried and weighed. Following redissolution in ethyl acetate, 3–5 mg of the Sep-Pak preparations was examined on a normal-phase silica high-performance liquid chromatography (HPLC) column with a refractive index detector.

As previously observed (Midland *et al.* 1993), *E. coli* cells expressing pAVRD12 (containing the *P. s.* pv. *tomato* class I allele of *avr*D) produced several unique peaks on HPLC that were not observed from cells with the cloning plasmid only (Fig. 1A). This HPLC profile is unique because of the presence of several doublets of peaks. The doublet of peaks 3 and 4 (called syringolides 2 and 1, respectively; Smith *et al.* [1993]) are the major elicitor-active compounds and are structurally identical, except that syringolide 1 (peak 4, Fig. 1) has an alkyl chain that is two carbons shorter. HPLC of preparations from *E. coli* cells expressing *avr*D allele 1 of *P. s.* pv. *lachrymans* also yielded a peak pattern very similar to that of the *P. s.* pv. *tomato* allele (data not shown). This is not surprising, since these alleles both belong to class I and are 95% identical at the amino acid level (Yucel and Keen 1994).

HPLC of preparations from *E. coli* cells expressing the class II *avr*D allele from *P. s.* pv. *phaseolicola* cloned in the high-expression plasmid pINIIIA-2 (Masui *et al.* 1984) (pPAD/RI) yielded a unique HPLC profile that lacked the families of doublet peaks characteristic of class I *avr*D alleles (Fig 1B). Only syringolide 1 (peak 4, Fig. 1B) was detected, with no trace of syringolide 2 (peak 3). Peak 2 (Fig. 1B) was shown by nuclear magnetic resonance (NMR) spectrometry to be identical to the corresponding peak from cells containing pAVRD12 (Fig. 1A), but peaks 1 and 5 were missing from cultures containing pPAD/RI. Peak 6 (Fig. 1B) yielded an NMR spectrum suggestive of two compounds, and this was confirmed by rechromatography on the same column with 8% isopropanol in hexanes (data not shown). Structures of the compounds in peaks 1, 2, and 5 and the two components in peak 6 are currently under investigation, but on the basis of NMR data, all of the compounds are related to the syringolides (S. Midland and J. Sims, unpublished observations). In addition, two as yet unidentified compounds (X and Y in Fig. 1B) were produced by bacteria expressing the *P. s.* pv. *phaseolicola avr*D allele but not the *P. s.* pv. *tomato* allele (Fig. 1A). Preparations from bacteria expressing *P. s.* pv. *lachrymans avr*D allele 2 or the mutant *P. s.* pv. *glycinea avr*D genes pRVS and pCAD5 (Yucel and Keen 1994) yielded HPLC peak patterns similar to those of the *P. s.* pv. *phaseolicola* allele, but the amounts produced were less than 5% of those produced by bacteria expressing pPAD/RI (data not shown). The lower production directed in *E. coli* by these

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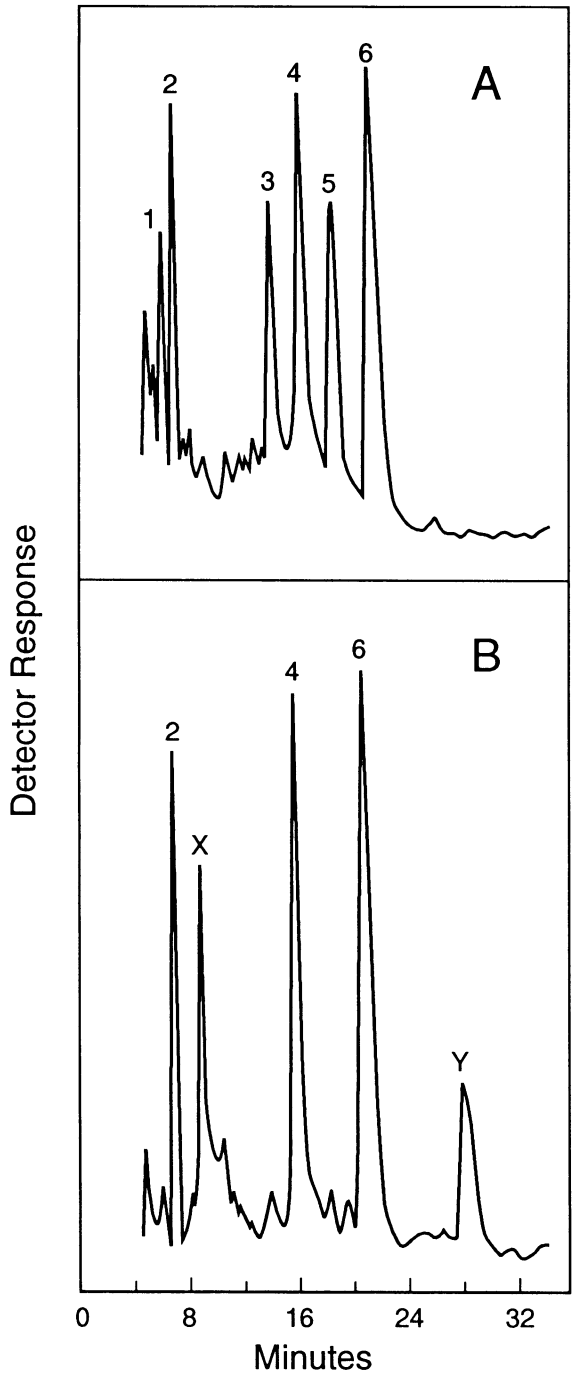

Fig. 1. High-performance liquid chromatography (HPLC) traces of processed culture fluids from *Escherichia coli* DH5α cells expressing the *Pseudomonas syringae* pv. *tomato* class I allele, pAVRD12 (**A**), or the *P. s.* pv. *phaseolicola avr*D class II allele, pPAD/RI (**B**). A 4.6 × 250 mm Phenomenex Maxsil 5 silica column was eluted isocratically with 40% ethyl acetate in hexanes at 1.5 ml/min, and a Waters refractive index detector was set at 4×. Ethyl acetate culture fluid extracts (3 mg) partially purified with a Sep-Pak silica cartridge were dissolved in about 20 µl of ethyl acetate and injected for each HPLC run. Processed culture fluids from 500 ml of *E. coli* culture carrying only the cloning plasmid, pINIII A-2 (Masui *et al.* 1984), yielded <1 mg of material from the Sep-Pak cartridge that did not produce any of the HPLC peaks shown.

constructs is assumed to result from the fact that the genes were expressed in pUC plasmids with weaker promoters than the pINIII A-2 constructs.

The 3.8-kb *PstI*/*SalI* DNA fragments carrying the expression cassettes with pINIII A-2 translational fusions of the *P. s.* pv. *tomato avr*D gene (from pAVRD12) or the analogous construct with the *P. s.* pv. *phaseolicola avr*D allele (from pPAD/RI) were cloned into pDSK519 and electroporated into *P. s.* pv. *glycinea* race 4. When these bacteria were grown on M9–casamino acids medium and the culture fluids were processed, HPLC peak patterns observed were similar to those seen when the respective alleles were expressed in *E. coli*. However, production of the various compounds was only about 20% that of the analogous constructs in *E. coli* (data not shown).

The nonfunctional *avr*D allele from *P. s.* pv. *glycinea* is a member of class II on the basis of its structure, but it does not give the avirulence D phenotype in soybean plants (Keen *et al.* 1990; Kobayashi *et al.* 1990b; Yucel *et al.* 1994; Yucel and Keen 1994). Although lacking avirulence gene activity, overexpression of the *P. s.* pv. *glycinea avr*D allele in *E. coli* results in extremely low levels of elicitor activity detectable by the soybean leaf bioassay (Keen *et al.* 1990). In the present work, processing 2 L of culture fluids from *E. coli* expressing various transcriptional and translational fusions of the *P. s.* pv. *glycinea avr*D allele did not result in detectable HPLC peaks corresponding to either of the syringolides or elicitor activity in soybean leaves. Thus, if this gene possesses the putative enzymatic activity of other class II alleles, it must be at an extremely low level. We recently defined amino acid residues in the *P. s.* pv. *glycinea avr*D allele that account for its lack of avirulence gene activity relative to functional class II alleles (Yucel *et al.* 1994; Yucel and Keen 1994). As discussed earlier, however, the mutationally repaired *P. s.* pv. *glycinea avr*D gene indeed led to elicitor activity and HPLC peaks characteristic of class II alleles. This confirmed the class II nature of the *P. s.* pv. *glycinea avr*D allele predicted from homology relationships.

The finding that the newly characterized class II *avr*D alleles yielded different products from class I alleles is of considerable significance. The results support our previous interpretation that the *avr*D protein products likely have enzymatic functions that lead to the elicitor-active syringolides as well as structurally related compounds of lesser activity. The *P. s.* pv. *tomato avr*D allele, when expressed in *E. coli* or in *P. s.* pv. *glycinea* race 4 cells, directed the production of compounds predicted to arise from the condensation of D-xylulose with either β-hydroxydecanoic acid (peaks 1, 3, and 5; Fig. 1A) or β-hydroxyoctanoic acid (peaks 2, 4, and 6; Fig. 1A and B) but not longer- or shorter-chain fatty acids (Smith *et al.* 1993). Some of the products produced by the newly characterized class II *avr*D alleles appear to arise exclusively from β-hydroxyoctanoic acid, but the corresponding products from β-hydroxydecanoic acid could not be detected. This indicates that the putative enzymatic functions of the two classes of *avr*D proteins discriminate against fatty acid derivatives differing in length by two carbon atoms. Thus, the utilization by class I *avr*D alleles of only β-hydroxyoctanoic and β-hydroxydecanoic acids is unlike normal biosynthetic enzymes of the malonyl CoA fatty acid biosynthetic pathway, which recognize intermediates irrespective of their chain

length. However, the class II *avr*D alleles appear to have an even more stringent substrate requirement, since they recognize the β-hydroxyoctanoic acid but not the β-hydroxydecanoic acid precursor.

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