Identification of new early markers of the hypersensitive response in *Arabidopsis thaliana*[1]

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Abstract New molecular markers of the hypersensitive response (HR) of *Arabidopsis thaliana* to the bacterial pathogen *Xanthomonas campestris* pv. *campestris* (*X.c.c.*.) have been identified by differential screening of a cDNA library constructed from suspension cells inoculated by an HR-inducing strain in the presence of cycloheximide. Seven families of genes (called *Athsr*) have been isolated, show similarities to voltage-dependent anion channels (VDAC) and alternative oxidases, or are novel proteins. *Athsr* genes have shown to be specifically or preferentially expressed during the HR. These data suggest that *Athsr* genes might be involved in early events conditioning the establishment of the HR.

Key words: Hypersensitive response; Cycloheximide; Differential screening; Voltage-gated ion channel protein; Alternative oxidase; *Arabidopsis*

1. Introduction

Infection of a plant by a pathogen results in one of two possible outcomes: disease (compatible interaction) or resistance (incompatible interaction). During an incompatible interaction, the plant cells at and adjacent to the site of infection, undergo rapid cell death, a phenomenon known as the hypersensitive response (HR). The HR is an active response requiring induction of a particular set of biochemical and molecular events. Changes in protein phosphorylation [1], reactive oxygen species (ROS) production [2], ion fluxes [3], and G-protein mediated signalling [4] are the fastest HR-associated events preceding cell death and the activation of so-called defense genes [5,6]. However, only a small number of genes involved in the regulation and execution of the HR cell death has been identified until now [7]. Although the function of their expression product is in most cases unknown [8–10], these genes whose activation is specific for the HR, are believed to play a role in the control of the HR cell death. Beside the isolation of mutants altered in the HR in response to pathogen attack, or presenting an uncontrolled cell death (lesion mimic mutants), the isolation of such genes might be an alternative way to identify important components of the pathway(s) leading to HR cell death.

In order to isolate HR-related early-induced genes in *Arabidopsis*, an original system has been developed in order to focus on genes whose expression does not depend on de novo protein biosynthesis. A differential screening of a cDNA library derived from *Arabidopsis* cell suspensions treated with cycloheximide (an inhibitor of protein synthesis) then infected with an HR-inducing strain of the pathogen *Xanthomonas campestris* pv. *campestris* (*X.c.c.*.) led to the isolation of genes called *Athsr* (*Arabidopsis thaliana* hyper-sensitivity-related).

2. Materials and methods

2.1. Plant material and bacterial strains

Cell suspension culture growth conditions, bacterial strains and inoculation procedures used in this study were as previously described [11].

2.2. Differential screening of the cDNA library and Northern blot analysis

All routine DNA and RNA manipulations were standard procedures [12]. For the cDNA library construction, total RNA was isolated from fresh cells 4 days after transfer to new culture medium, preincubated for 1 h with 1 μM cycloheximide and inoculated with the avirulent strain of *X.c.c.*. (strain 147), as previously described [11]. RNA extraction and Northern analyses were done as previously described [11,13].

2.3. DNA sequencing and nucleotide sequence analysis

Nucleotide sequencing and sequence analysis was carried out as previously described [11,14]. Sequence alignments were generated using the BOXSHADE program.

3. Results and discussion

3.1. Isolation by differential screening and sequence analysis of early HR-induced cDNAs (*Athsr*)

To isolate genes showing preferential expression during the very early steps of HR, a cDNA library of *A. thaliana* suspension cells infected for 1 h by a corresponding avirulent *X.c.c.* isolate in absence of protein synthesis, was constructed and subjected to a differential screening using probes from cells inoculated either with an *hrp*-deletion mutant of *X.c.c.*. (strain 8B2, asymptomatic) or the HR-inducing strain 147 (Fig. 1). After four successive rounds of differential screening, a total of 27 independent cDNA clones were isolated, and called *Athsr*. Cross-hybridization experiments and systematic partial sequencing indicated that they correspond to seven different families of genes with different relative abundance of cDNA clones (Table 1).

A whole cDNA insert was sequenced for a representative of the two major cDNA families isolated (*Athsr2* and *Athsr3*), or partially sequenced for the other cDNA families, and the DNA sequences subjected to database homology search indicated similarities to known plant proteins for some of them (Table 1). The *RaR121* clone (Fig. 2A), a representative of the *Athsr2* family, was found to be highly similar to mitochondrial voltage-dependent gated anion channel (VDAC) pro-

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[1] The Genbank accession numbers for the sequences reported in this paper are AJ131391 (*Athsr2*), AJ131392 (*Athsr3*), AJ243377 (*Athsr4*), AJ243378 (*Athsr5*), AJ243379 (*Athsr6*) and AJ243380 (*Athsr7*).

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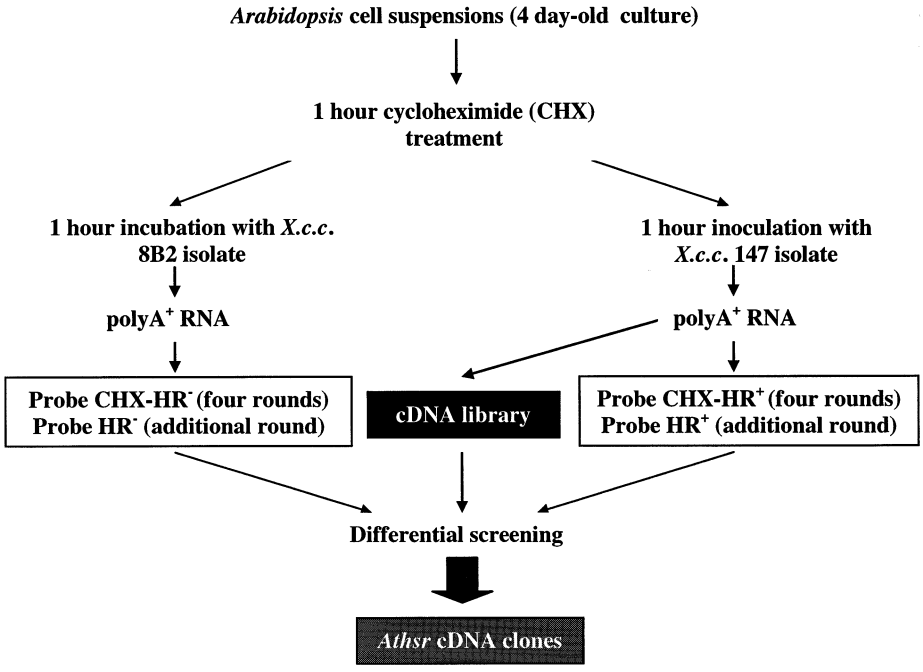

Fig. 1. Cloning strategy of early-induced HR genes (*Athsr*) from *Arabidopsis* cell suspensions. The complex probes generated from cell suspensions inoculated with the HR-inducing strain from *X.c.c.* isolate 147 (*Xcc* 147) or with the non-symptomatic isolate (*Xcc* 8B2), with or without cycloheximide (CHX), were used for differential screening of the cDNA library.

teins, previously isolated in other plant species [15,16] (Fig. 2A). VDAC are voltage-gated diffusion pores located in the outer membrane of mitochondria. They play a crucial role in regulating the transmembrane potential of mitochondria [17], the release of ions [18] or other small molecules (cytochrome *c*), that are key events during apoptosis in mammalians [17,19]. Until now, *Athsr2* remains the only example of VDAC isolated from *Arabidopsis* and furthermore its original induction pattern during HR provides new clues for the function of this protein family in regulating cell death processes. The *RaR105* clone that belongs to the *Athsr3* family (Fig. 2B) is similar to alternative oxidase proteins isolated from other plant species [20–22]. These proteins are involved in the cyanide-insensitive respiratory pathway in mitochondria, which is operative when the conventional cytochrome pathway is either constricted by respiratory inhibitors or saturated with an excess of electrons occurring during stress [20,23]. This alternative pathway has been recently proposed to be involved in resistance mechanisms to pathogens [24]. The last four clones (*Athsr4*, *Athsr5*, *Athsr6* and *Athsr7)* were not seemingly related to known DNA or protein sequences and probably encode novel proteins. However, one cDNA clone that cross-hybridizes with *Athsr4* cDNA is similar to *A. thaliana* *rab*GDP dissociation inhibitor (*rab*GDI) protein [25], a large protein family regulating gene expression and recently associated with induction of salicylic acid in response to wounding [26]. Due to the rapid induction of *Athsr4* during HR, *Athsr4* could be part of a fine tuning gene regulation mechanism in response to pathogen attack. The three last classes of *Athsr* cDNAs should represent novel genes whose expression levels

Table 1
Sequence homologies detected for *Athsr* clones

Clone family	Number of clones isolated	Insert size	Clones sequenced	Best homology	Statistical significance
Athsr2	16	from 1kb to 1.5 kb	*RaR121* (complete 1108 bp)	Voltage-dependent anion selective channel (POM34) (*S. tuberosum*)	69% identical; 81% similar on 274 amino acids (GAP-GCG) $P = 1.2 \times 10^{E} - 133$ (BLASTX)
			partial 5′- and 3′-ends for the 15 other clones		
Athsr3	6	from 1.4 kb to 3 kb	*RaR105* (complete 1360 bp)	Alternative oxidase AO×1A protein (*A. thaliana*)	94% identical; 96% similar on 353 amino acids (GAP-GCG) $P = 1.9 \times 10^{E} - 247$ (BLASTX)
			partial 5′- and 3′- ends for the 5 other clones		
Athsr4	1	2.5 kb	*Athsr4* 364 bases, 5′-end	none	
			RaR141 (cross-hybridization with *Athsr4)* 224 bases, 5′-end	GDP dissociation inhibitor (*A. thaliana*)	95% identical; 95% similar on 46 amino acids $P = 4.8 \times 10^{E}$-24 (BLASTX)
Athsr5	1	0.9 kb	137 bases, 5′-end	none	
Athsr6	1	3 kb	95 bases, 5′-end	none	
Athsr7	1	1.6 kb	125 bases, 5′-end	none	

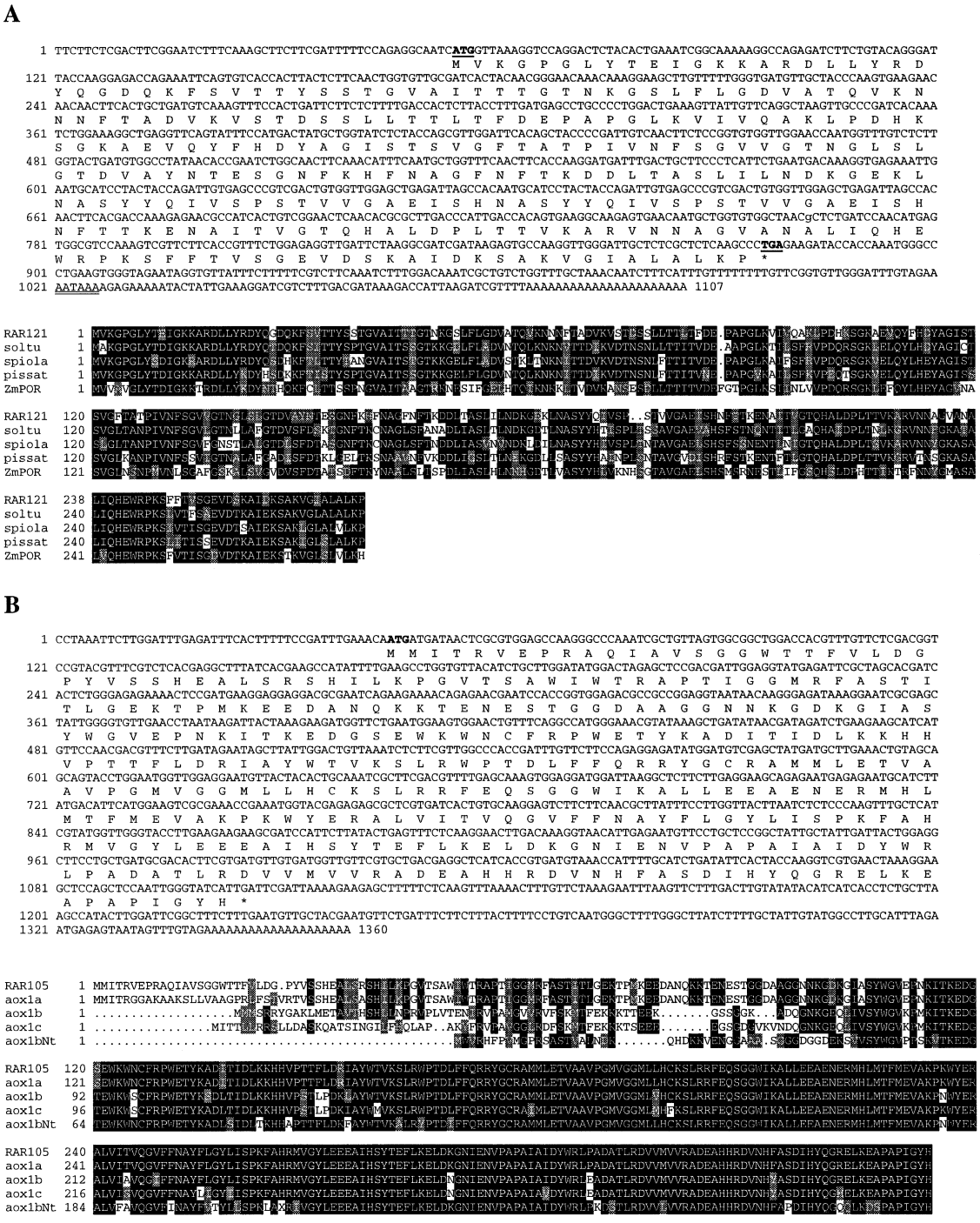

Fig. 2. Sequence analysis of cDNA clones from *Athsr2* and *Athsr3* families. A: Nucleotide and deduced amino acid sequences from RaR121 cDNA clone belonging to *Athsr2* family. The ATG translation initiation codon and TGA termination codon are shown in bold character. The putative polyadenylation signal sequence is underlined. Multiple sequence alignment of RaR121 deduced primary structure with related proteins from different plant species is shown below: *Solanum tuberosum* (Soltu, *Pom34* GB:X80386), *Spinacia olaracea* (Spiola, *SVDAC1* GB:U50900), *Pisum sativum* (Pissat, *POR1* GB:Z25540), *Zea mays* (ZmPOR, *POR1* GB:X73429). The amino acids conserved or are homologous for at least three proteins are in black or gray boxes respectively. B: Nucleotide and deduced amino acid sequences from the RaR105 cDNA clone belonging to the *Athsr3* family. The ATG translation initiation codon and TGA termination codon are shown in bold character. The putative polyadenylation signal sequence is underlined. Multiple sequence alignment of RaR105 deduced primary structure with related alternative oxidases from different plant species are shown below: *A. thaliana* (*aox1a* and *aox1b* GB:D89875, *aox1c* GB:AB003175) and *Nicotiana tabacum* (*aox1bNt* GB:X79768). The amino acids conserved or belonging to the same family in at least four proteins are in black or gray boxes, respectively.

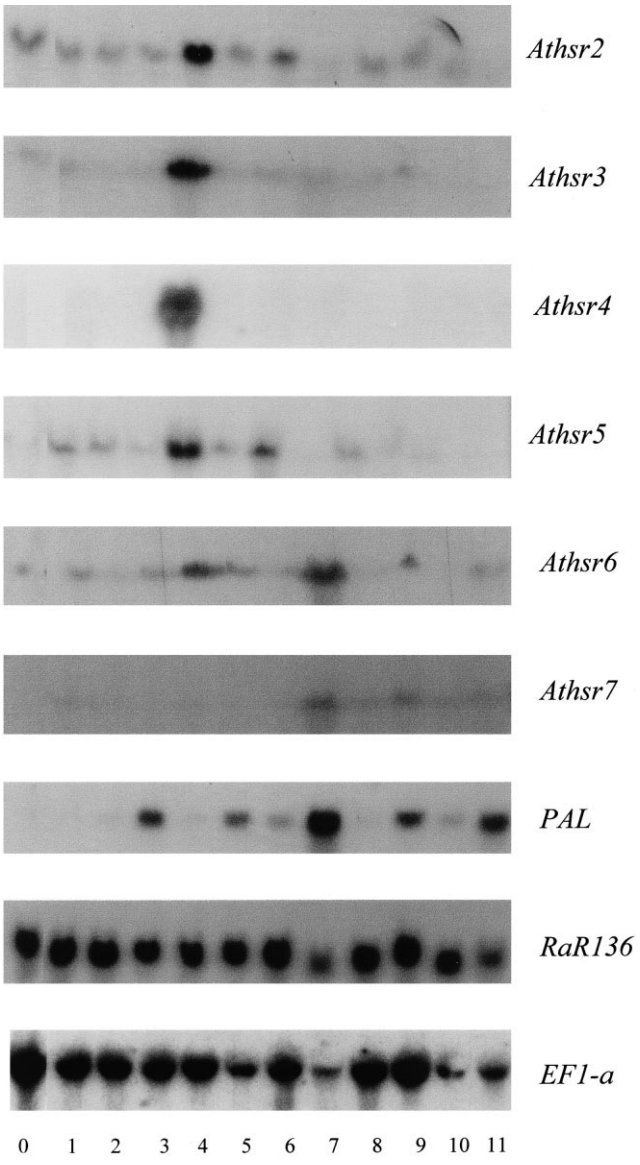

Fig. 3. Expression analysis of representative *Athsr* clones. Northern analysis of total RNA isolated from cell suspensions at different times after inoculation (1, 3, 6 and 24 hpi) with different strains of *X.c.c.* : a non-symptomatic strain (8B2) (lanes 1, 2 and 3) corresponding to 3, 6 and 24 hpi), an avirulent strain (147) (lanes 4, 5, 6 and 7 corresponding to 1, 3, 6 and 24 hpi) and a virulent strain (8004) (lanes 8, 9, 10 and 11 corresponding to 1, 3, 6 and 24 hpi). The left lane, labelled 0, represents a control, which corresponds to RNA prepared from non-inoculated cells at time 0 of the experiment. The blot was sequentially hybridized with cDNA probes as indicated to the left of each panel. Autoradiography exposures vary, depending on the clone, from 1 to several days.

are related as a cause or a consequence of HR establishment. In addition to these *Athsr* cDNA clones, we have isolated another *Athsr* gene family (*Athsr1*) encoding a putative transcription factor, whose characteristics are described elsewhere [27].

3.2. *Expression patterns of Athsrs in suspension-cells inoculated with different isolates of Xanthomonas campestris pv. campestris*

To study the expression of *Athsr* corresponding genes in response to avirulent or virulent *X.c.c.*. isolates, cell suspension cultures were inoculated by isolates 8B2 (control, *hrp* deletion mutant), 147 (HR-inducing strain) or 8004 (virulent strain, inducing disease) for different periods of time. Fig. 3 shows that mRNA levels of *Athsr2*, *Athsr3* and *Athsr4* increase transiently within the first hour during the HR. *Athsr5*, *Athsr6* and *Athsr7* transcripts show a less marked preferential accumulation during HR, with a maximum at 1, 6 or 24 h during HR. For comparison, *PAL* transcripts accumulate during both the HR and the compatible interaction, as previously demonstrated in other pathosystems [28]. Probing with the clone *RaR136*, previously used as an internal control during the differential screening of the cDNA library (same hybridization signal level with probes CHX-HR$^-$ and CHX-HR$^+$, data not shown) indicate that the mRNA accumulation levels are constant during the time-course of infection. Similarly, *EF1-α* expression remains stable showing however a significant decrease 24 h after inoculation both with the avirulent and the virulent *X.c.c.* isolates probably due to a loss of viability of the suspension cells.

Isolation of genes induced early and specifically during the HR remains a prerequisite to the understanding of resistance mechanisms to pathogen attack and to elucidate fundamental processes involved in gene regulation preceding programmed cell death (PCD) occurring during the HR [29]. Plant defense gene activation is an important element in the outcome of plant-pathogen interaction [7–10], and the identification of *Athsr* genes, their characteristics of activation during the HR and their putative functions make them good candidates for modulating early events of plant cell death programs. Functional studies of the *Athsr*s gene product by sense-antisense approaches in transgenic plants and using plant virus-based expression vector will be developed. They should bring more information about the role of *Athsr* genes in regulatory pathways leading to the HR cell death and/or to resistance to pathogens.

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