Eur J Plant Pathol (2008) 122:307–314
DOI 10.1007/s10658-008-9294-1

Transcript profiling for *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent defence gene expression

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Received: 30 November 2007 / Accepted: 18 February 2008 / Published online: 8 March 2008

Abstract Tomato *Cf* genes confer resistance to the leaf mold pathogen *Cladosporium fulvum*. The *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent hypersensitive responses (HRs) are distinct in cell death pattern, intensity, and sensitivity to environmental conditions. To understand the mechanism resulting in these differences, comparative transcript profiling for *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent defence gene expression by cDNA-AFLP was performed previously. 367 *ACE* (*Avr/Cf-elicited*) transcript-derived fragments (TDFs) were identified, among which 189 were cloned and sequenced. In this study, we report another 89 *ACE* fragments. These *ACE* genes were associated with: defence, signal transduction, HR and cell death, transcriptional regulation, metabolism, protein synthesis, photosynthesis, membrane fusion, secretion and trafficking, miscellaneous biological processes, and genes with unknown function or with no significant similarity to known sequences. Among these sequences 43 (potentially encoding 36 types of proteins) were identified for the first time as genes differentially expressed during the development of *Avr/Cf*-dependent HR. Sequence and expression data from this study further support that transcription is reprogrammed to promote defence response and HR and repress photosynthesis in the *Avr/Cf* HR^+ seedlings.

Keywords *ACE* · *Cladosporium fulvum* · Defence · Signal transduction · Tomato · Transcriptome

Cladosporium fulvum is the fungal pathogen of tomato leaf mould disease. The tomato and *C. fulvum* pathosystem is a model system for the study of gene-for-gene resistance (Joosten and De Wit 1999; Wang et al. 2006). From this pathosystem, several *Avr* genes and the cognate *Cf* genes, including *Avr9/Cf-9* (Van den Ackerveken et al. 1992; Jones et al. 1994), and *Avr4/Cf-4* (Joosten et al. 1994; Thomas et al. 1997), have been cloned. Both the *Cf-4* and *Cf-9* genes encode extracellular, membrane-anchored, glycoproteins that consist mainly of LRR domains (Jones et al. 1994; Thomas et al. 1997). Furthermore, over 91% of the amino acids of the Cf-4 and Cf-9 proteins are identical (Jones et al. 1994; Thomas et al. 1997). However, the hypersensitive response (HR) resulting from recognition of Avr9 and Avr4 by Cf-9 and Cf-4, respectively, is distinct in cell death pattern and

Electronic supplementary material The online version of this article (doi:10.1007/s10658-008-9294-1) contains supplementary material, which is available to authorized users.

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intensity (Cai et al. 2001). Thus, compared to the *Avr9/Cf-9*-dependent HR, *Avr4/Cf-4*-dependent HR is more rapid (Thomas et al. 2000; Van der Hoorn et al. 2000; Cai et al. 2001), with necrosis appearing primarily in the veins of the *Avr/Cf*-carrying F_1 seedlings resulting from crosses between Cf plants and Cf0 plants expressing a complementary *Avr* gene (Cai et al. 2001). In addition, the two *Avr/Cf*-dependent HRs are different in their sensitivity to environmental conditions. *Avr9/Cf-9*-dependent HR is more sensitive to high temperature and high relative humidity than *Avr4/Cf-4*-dependent HR (De Jong et al. 2002; Wang et al. 2005).

To elucidate the mechanism leading to the distinct nature of the *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent HRs, we have previously compared the defence signal transduction pathways and the resulting defence response downstream of Cf-4 and Cf-9 by comparative transcript profiling of *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent defence gene expression employing cDNA-AFLP analysis of F_1 hybrid tomato lines expressing the gene pair *Avr4/Cf-4* or *Avr9/Cf-9* (Hong et al. 2007). 367 *ACE* (*Avr/Cf-elicited*) transcript-derived fragments (TDFs), which showed significant differential expression between HR^+ and HR^- seedlings (either *Avr4/Cf-4*- or *Avr9/Cf-9*-dependent), were identified. The expression data reveal that the spectrum of the genes differentially expressed during HR development is most likely to be identical for both *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent HRs. However, a significant number (42.8%) of the *ACE* TDFs showed quantitatively different expression in the two types of HR^+ seedlings. The majority of these (86.0%) displayed significantly greater differential expression (either induced or repressed) in *Avr4/Cf-4* HR^+ seedlings than in *Avr9/Cf-9* HR^+ seedlings (Hong et al. 2007). These results are consistent with the earlier observation that *Avr4/Cf-4*-dependent HR is more severe than *Avr9/Cf-9*-dependent HR (Cai et al. 2001).

Among the 367 *ACE* TDFs, 189 fragments, which displayed the most significant differences in the expression level between the HR^+ and HR^- samples have been cloned and sequenced in our previous study (Hong et al. 2007). In the present study, more insights into the mechanism of the *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent HRs were provided, and another 89 *ACE* TDFs, which showed differential expression between the HR^+ and HR^- samples, were cloned and sequenced.

The dried polyacrylamide gels with separated cDNA-AFLP selective PCR products, conducted previously (Hong et al. 2007), were used to obtain new *ACE* sequences. The fragments corresponding to genes differentially expressed in the HR^+ *Avr/Cf* seedlings (8 h after temperature shift from 33°C to 20°C when clear hypersensitive necrosis is visible by the naked eyes) in comparison with the HR^- *Avr/Cf* seedlings (grown constantly at 33°C) and the *Cf* and *Avr* parent seedlings (either grown constantly at 33°C or after a temperature shift) were subjected to cloning. The bands containing differentially expressed fragments, amplified from the *Avr4/Cf-4* seedlings (or from the *Avr9/Cf-9* seedlings, in cases where they were more strongly expressed in this type of seedlings), were excised from the dried gels and eluted in 100 μl of 2 mm Tris–HCl (pH 8.0) overnight at room temperature, kept in a water bath at 55°C for 10 min, after which 5 μl was re-amplified using the same primer set as for the initial selective PCR amplification. The fragments were cloned into pGEM-T easy vector (Promega, USA) and sequenced. In this way, a total of 89 sequences was obtained. Among them, 56 were unique, while the other 33 corresponded to 11 TC or SGN-U sequences. Therefore, the maximum number of newly-cloned *ACE* genes corresponding to the 89 fragments was 67.

The sequences were analysed for homology by searching in GenBank, DFCI (Dana Farber Cancer Institute, http://compbio.dfci.harvard.edu/tgi) and SGN (the sol genomics network, http://www.sgn.cornell.edu/) databases using the BLAST sequence alignment programmes (Altschul et al. 1997; Gish 1996–2006, http://blast.wustl.edu). Considering a great number of tomato EST and TC sequences ('Tentative Consensus' sequences, which are assemblies of non-human ESTs) are available in the DFCI and SGN databases, we first used the obtained 89 *ACE* sequences to search for homologues deposited in these databases by BLASTN analyses, which were then used to search for homologues deposited in the GenBank database by BLASTX analyses. In cases where no homologues were retrieved in the DFCI and SGN databases, direct BLAST searches were performed in the GenBank database for the *ACE* sequences.

Results of homology searches revealed that out of the 89 newly-cloned *ACE* fragments, 1 (1.1%) had no significant similarity to known sequences (*E*-value

Table 1 Functional classification of the 89 *ACE* TDFs cloned in this study

Functional classes	*ACE* fragments	Percentage (%) of the 89 fragments
HR/cell death-associated	1	1.1
Signalling-related	9	10.1
Defence-related	9	10.1
Transcriptional regulation	9	10.1
Metabolism	13	14.7
Protein synthesis	8	9.0
Photosynthesis	18	20.3
Miscellaneous	9	10.1
Membrane fusion and secretion	1	1.1
Membrane trafficking	1	1.1
Stress-responsive	1	1.1
Unknown function	9	10.1
No similarity to known sequences	1	1.1
Total	89	100

>0.05); 9 (10.1%) were homologous to sequences with an unknown function, while the remainder (79, 88.8%) had homology to sequences with known functions such as defence and resistance, signal transduction, HR and cell death, transcriptional regulation, metabolism, protein synthesis, membrane fusion, secretion and trafficking, photosynthesis and miscellaneous biological processes (Supplementary Table 1; Table 1).

The largest class of the 89 *ACE* fragments was related to sequences involved in photosynthesis. This class contained 18 fragments, corresponding to 20.2% of the total 89 sequences. The encoding products of this class of *ACE* genes comprised 10 distinct types of proteins, including chlorophyll biosynthetic enzymes magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase and Mg-protoporphyrin IX chelatase, light harvesting chlorophyll a/b-binding proteins, a set of photosystem I and II reaction centre subunits and assembly proteins, electron transporter ferredoxins, and CO_2 assimilation regulator ribulose bisphosphate carboxylase subunits. All these *ACE* genes were down-regulated in HR^+ seedlings when compared with HR^- seedlings, demonstrating that photosynthesis is repressed in the HR^+ seedlings. This observation is consistent with our earlier result (Hong et al. 2007).

The metabolism-related class of *ACE* fragments is the second largest, containing 13 fragments (14.6%), most of which were associated with synthesis, modification and transportation of nutrients and secondary metabolites, among which many were involved in plant defence responses.

There were nine defence-related *ACE* fragments, which corresponded to genes encoding a carbonic anhydrases, a xyloglucan-specific fungal endoglucanase inhibitor protein, a polyphenol oxidase, a methionine-rich arabinogalactan protein, and the well-known defence-related proteins such as β-1, 3-glucanase, endochitinase and tomato pathogenesis-related protein P2. Additionally, one *ACE* fragment corresponded to the gene encoding a Hin1-like protein, which is a well-known HR marker.

Nine signalling-related *ACE* fragments were identified. Seven corresponded to the same gene encoding a GTP-binding protein, while the other two corresponded to genes encoding a nodulin-like protein and a calcium-binding EF hand family protein, respectively.

Nine *ACE* fragments corresponded to genes encoding for at least four types of transcriptional regulators: ethylene-responsive ER33 protein/BHLH transcription factor, C2H2-type zinc finger family transcriptional factor, DEAD-box RNA helicase-like protein, and RNA polymerase subunits. The class of protein synthesis-related *ACE* fragments, totally eight, corresponded to genes encoding a set of ribosomal proteins.

An *ACE* fragment matched the gene encoding a syntaxin-like protein, which is involved in membrane fusion and secretion, while another corresponded to the gene encoding beta prime of a coatomer protein complex (COP), which plays a role in membrane trafficking.

Additionally, one group of the *ACE* fragments corresponded to genes associated with multiple biological processes, including tubby-like F-box protein, proton-dependent peptide transport family protein, C2 domain-containing protein, trigger factor-type chaperone family protein, ABC transporter ATPase, and a set of proteins involved in protein degradation, activation and modification, such as ATP-dependent Clp protease and DnaJ protein.

Transcript profiling of *Avr/Cf*-dependent HR has been conducted previously using cDNA-AFLP analysis. TDFs of *ACRE* (for *Avr9/Cf-9* rapidly elicited) genes and *ART* (for Avr4-responsive tomato) genes have been cloned (Durrant et al. 2000; Gabriëls et al. 2006). Therefore, analysis of sequence overlap for *ACRE*, *ART* and our previously cloned *ACE* fragments

(Hong et al. 2007) was performed using DNAstar SeqMan II software. The *ACE*-matching TC and SGN sequences were also included together with *ACRE*, *ART* and our previously cloned *ACE* fragments for assembly analysis. We found that of the 89 *ACE* fragments cloned in this study, 43 fragments (potentially encoding 36 types of proteins) were distinct from the reported Avr/Cf elicited sequences (Table 2). They are involved in a wide range of functions (Table 2) and provide new information potentially useful in elucidating the mechanism of the *Cf/Avr*-dependent HR and resistance.

Arabinogalactan proteins (AGPs) are a family of highly glycosylated, hydroxyproline-rich glycoproteins implicated in plant growth and development, hormone signalling and programmed cell death (Chaves et al. 2002). Accumulation of an AGP protein, attAGP, in host plant tomato at the sites of attack by parasitic plant dodder promotes the parasite's adherence, and thus is beneficial for its infection (Albert et al. 2006). In this study we found that a tomato *AGP* gene (*ACE344*) is down-regulated during the development of *Cf/Avr*-dependent HR.

Among the three unique transcription-related ACE proteins is a DEAD-box RNA helicase-like protein. The DEAD-box RNA helicases comprise the largest subfamily of RNA helicases. They play regulatory roles in transcription, translation, RNA processing and ribosome assembly (Lorsch, 2002). Some specific functions of these proteins in plants, such as the biogenesis of microRNAs and plant development (Jacobsen et al. 1999; Park et al. 2002) and cold

Table 2 Newly cloned *ACE* TDFs distinct from *ART*, *ACRE* and *ACE* TDFs cloned before

Functional class	Product	*ACE* fragment
Defence-related	Methionine-rich arabinogalactan protein	344
Signalling-related	Nodulin-like protein	321a
Transcriptional regulator	BHLH transcription factor/Ethylene-responsive ER33 protein	171,172
	RNA polymerase subunit	305,311
	DEAD-box RNA helicase-like protein	331
Metabolism	Acetyl-CoA synthetase	272
	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	277
	Glyceraldehyde-3-phosphate dehydrogenase B subunit	290
	Nicotianamine synthase	292
	Flavonol synthase-like protein	309
	Reticuline oxidase precursor/Nectarin 5-like protein	333
	Chloroplast inorganic pyrophosphatase	358b
Photosynthesis	Photosystem I assembly protein	322a,322c
	Membrane-associated 30 kDa protein chloroplast precursor	334
	Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase chloroplast precursor	251
	Mg-protoporphyrin IX chelatase	345
	Tetratricopeptide repeat (TPR) domain containing ferredoxin-like protein	348
	Ferredoxin-1 chloroplast precursor	351
Miscellaneous	Proton-dependent peptide transport family protein	281
	C2 domain-containing protein	285b
Stress-responsive	Stress enhanced protein 2	289
Membrane fusion and secretion	Syntaxin 132-like	267
Membrane trafficking	Coatomer protein complex beta prime	273
Protein synthesis	Chloroplast 30S ribosomal protein	258a,347,358a
	40S ribosomal protein S8	276
	Plastid ribosomal protein S10 precursor	346,361a
Unknown	Expressed unknown protein	263a,274,284,288,295,314,337,352,252a
	No similarity to known sequences	161

response (Gong et al. 2005) have been reported. Mutations of the allelic DEAD-box RNA helicase genes *cryophyte/los4-2* and *los4-1* both affect cold responses but in opposite ways (Gong et al. 2005). It is still unknown whether the DEAD-box RNA helicase is involved in plant defence. In present study we found that a tomato DEAD-box RNA helicase-like protein gene (*ACE331*) is strongly down-regulated during the development of *Cf/Avr*-dependent HR. It will be interesting to further investigate its possible role in this process.

Up-regulation of a gene encoding a syntaxin 132-like protein (ACE267), in *Avr/Cf* HR^+ seedlings was observed in this study. Syntaxins are essential components of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) machinery, controlling vesicle trafficking in cells (Sanderfoot et al. 2001). In plants, some plasma membrane syntaxins such as SYP121 and SYP132 have been found to be essential for defence and disease resistance (Collins et al. 2003; Kalde et al. 2007; Zhang et al. 2007). A syntaxin identified from *Nicotiana benthamiana*, NbSYP132, is a key component contributing to *AvrPto/Pto*-mediated gene-for-gene resistance, and basal and salicylate-associated defense to bacterial pathogens, possibly via regulating exocytosis of vesicles containing antimicrobial PR proteins (Kalde et al. 2007). Furthermore, a tobacco syntaxin NtSyp121 is rapidly phosphorylated after Avr9 elicitation (Heese et al. 2005). These data indicate that syntaxin-mediated membrane trafficking may play an important role in *Cf*-dependent HR and resistance.

Two unique *ACE* fragments correspond to genes involved in miscellaneous biological processes. They encode a proton-dependent peptide transporter family protein (ACE281) and a C2 domain-containing protein (ACE285b). The peptide transporter family (PTR) proteins can transport peptides as well as many other molecules (Stacey et al. 2002). Recently, an *Arabidopsis* peptide transporter AtPTR3 was found to be required for defence against virulent pathogenic bacteria *Erwinia carotovora* subsp. *carotovora* and *Pseudomonas syringae* pv. *tomato* (Karim et al. 2007). The C2 domain is a Ca^{2+}-dependent membrane-targeting module found in many cellular proteins involved in a variety of biological processes such as signal transduction or membrane trafficking. The C2 domain-containing proteins BON1, BAP1 and BAP2 were reported to form into a complex to negatively regulate both basal and the *R* gene *SNC1*-mediated defence to bacterial and oomycete pathogens and act as inhibitors of programmed cell death (Yang et al. 2006, 2007). We observed that *ACE281* is up-regulated while *ACE285b* is down-regulated during the *Cf/Avr*-dependent HR. These expression patterns are positively correlated to HR and defence development, indicating their possible involvement in the process.

In addition to the 43-unique *ACE* fragments, the remaining 46 (encoding 26 types of proteins), involved in a variety of functions, are functionally overlapped by *ACRE*, *ART*, and/or our previously identified *ACE* genes (Supplementary Table 2). These include Zinc finger (C2H2 type) family DNA/RNA binding protein, carbonic anhydrase, GTP binding protein, calcium binding protein, Hin1-like protein, and a group of proteases (Supplementary Table 2).

Dissection of the functions of the *ACE* genes mentioned above, as well as those with unknown function or with no similarity to known sequences, will provide new information on the possible mechanism of the *Cf/Avr*-dependent HR and resistance.

One of the aims of our transcript profiling study is to examine whether the *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent signalling pathways and defence responses are different. Combining the sequence data of this study and the previous one, we found that the two Avr/Cf interactions elicit expression change of the same *ACE* genes (Hong et al. 2007; this study). However, among the total 278 cloned *ACE* fragments, 107 (38.5%) displayed different expression levels in the *Avr9/Cf-9* HR^+ and *Avr4/Cf-4* HR^+ seedlings. Of these, 91 (85.1%) had higher expression levels in the *Avr4/Cf-4* HR^+ seedlings. These 91 *ACE* fragments are involved in a variety of functions including defence, HR and cell death, signal transduction, and transcriptional regulation (Table 3). These data are consistent with our previous observations that the *Avr4/Cf-4*-dependent HR in *Avr/Cf* tomato seedlings is more rapid and severe than the *Avr9/Cf-9*-dependent HR (Cai et al. 2001), and further indicate that the *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent signalling pathways are similar, and thus the different cell death patterns of the *Avr4/Cf-4*- and *Avr9/Cf-9*-dependent HR probably result from events upstream of signal transduction and activation of defence responses, such as a different level and/or tissue specificity of *Cf* gene expression and/or a different Avr recognition mechanism by the two Cfs.

Table 3 Functional classification and expression of the total 278 *ACE* TDFs

Functional classes	*ACE* fragments	Percentage (%) of the 278 fragments	*ACE* fragments differentially expressed when elicited by *Avr9/Cf-9* and *Avr4/Cf-4*[a]	*ACE* fragments expressed more strongly when elicited by *Avr9/Cf-9*[b]	*ACE* fragments expressed more strongly when elicited by *Avr4/Cf-4*[c]
Resistance protein	1	0.4	1 (100%)	0	1 (100%)
HR/cell death-associated	11	4.0	7 (63.6%)	0	7 (100%)
Signalling-related	23	8.3	9 (39.1%)	3 (33.3%)	6 (66.7%)
Defence-related	71	25.5	21 (29.6%)	2 (9.5%)	19 (90.5%)
Transcriptional regulation	15	5.4	7 (46.7%)	1(14.3%)	6 (85.7%)
RNA splicing	2	0.7	0	0	0
Protein and water transport	4	1.4	2 (50%)	0	2 (100%)
Metabolism	53	19.1	21 (40.4%)	2 (9.5%)	19 (90.5%)
Protein synthesis	13	4.7	7 (53.8%)	1 (14.3%)	6 (85.7%)
Photosynthesis	29	10.4	6 (20%)	1 (16.7%)	5 (83.3%)
Miscellaneous	21	7.5	9 (42.8%)	2 (22.2%)	7 (77.8%)
Membrane fusion and secretion	1	0.4	1(100%)	0	1(100%)
Membrane trafficking	1	0.4	0	0	0
Stress-responsive	1	0.4	0	0	0
Unknown function	29	10.4	15 (51.7%)	4(26.7%)	11 (73.3%)
No similarity to known sequences	3	1.1	1 (100%)	0	1 (100%)
Total	278	100	107 (38.5%)	16 (14.9%)	91 (85.1%)

[a] The percentage *ACE* fragments differentially expressed when elicited by the *Avr9/Cf-9* and *Avr4/Cf-4* in total *ACE* fragments of this class is given in parenthesis.

[b] The percentage *ACE* fragments expressed more strongly when elicited by the *Avr9/Cf-9* in total *ACE* fragments differentially expressed when elicited by the *Avr9/Cf-9* and *Avr4/Cf-4* is given in parenthesis.

[c] The percentage *ACE* fragments expressed more strongly when elicited by the *Avr4/Cf-4* in total *ACE* fragments differentially expressed when elicited by the *Avr9/Cf-9* and *Avr4/Cf-4* is given in parenthesis.

A variety of molecular and physiological processes are affected by transcriptional regulation in the *Avr/Cf* HR^+ seedlings. The major physiological processes include defence response, metabolism, respiration and biological oxidation, and photosynthesis (Supplementary Table 1 of Hong et al. 2007; Supplementary Table 1 and Table 3 of this study). As expected, defence response appears to be a major focus of transcriptional regulation, with this class of *ACE* fragments comprising 25.5% of the total cloned 278 sequences. Metabolism also dramatically changes in the *Avr/Cf* HR^+ seedlings in comparison with the HR^- seedlings. This class of *ACE* fragments compose 19.1% of the 278 sequences. Many metabolism-related *ACE* genes encode enzymes and proteins that are essential for biosynthesis of defence- and HR-related molecules; and their expression patterns are beneficial for accumulation of defence- and HR-related compo-

nents, indicating that metabolism in the *Avr/Cf* HR^+ seedlings is reprogrammed to promote defence response and HR. Some examples are *ACEs* 149, 199, 200, 268, 272, 277, which correspond to genes encoding a glycosyltransferase, an auxin and ethylene-responsive GH3-like protein, and an acetyl-CoA synthetase, respectively, which have been reported to play a role in plant defence and resistance (Langlois-Meurinne et al. 2005; Nobuta et al. 2007; Tang et al. 2007). Additionally, over 10% of the 278 *ACE* fragments correspond to genes encoding numerous components of a complex involved in the whole process of photosynthesis (Supplementary Table 1 of Hong et al. 2007; Supplementary Table 1 and Table 3 of this study). These biological processes are affected differently in the *Avr/Cf* HR^+ seedlings: defence response, respiration and biological oxidation are strongly induced while photosynthesis is tremendously repressed, as indicated by the change of expression of the related *ACE* genes (Supplementary Table 1 of Hong et al. 2007; Supplementary Table 1 of this study).

Acknowledgements We are grateful to Dr. Matthieu Joosten (Wageningen University, The Netherlands) for providing sequences of the *ART* fragments. This work was financially supported by the National Basic Research Programme of China (grant No. 2006CB101903), the Fok Ying Tong Education Foundation (grant No. 101032), and the National Natural Science Foundation of China (grant nos. 30070492, 30671352).

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