

# Fusicoccin, 14-3-3 Proteins, and Defense Responses in Tomato Plants<sup>1</sup>

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**Fusicoccin (FC) is a fungal toxin that activates the plant plasma membrane H<sup>+</sup>-ATPase by binding with 14-3-3 proteins, causing membrane hyperpolarization. Here we report on the effect of FC on a gene-for-gene pathogen-resistance response and show that FC application induces the expression of several genes involved in plant responses to pathogens. Ten members of the FC-binding 14-3-3 protein gene family were isolated from tomato (*Lycopersicon esculentum*) to characterize their role in defense responses. Sequence analysis is suggestive of common biochemical functions for these tomato 14-3-3 proteins, but their genes showed different expression patterns in leaves after challenges. Different specific subsets of 14-3-3 genes were induced after treatment with FC and during a gene-for-gene resistance response. Possible roles for the H<sup>+</sup>-ATPase and 14-3-3 proteins in responses to pathogens are discussed.**

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The plant plasma membrane H<sup>+</sup>-ATPase governs the electrochemical gradient across the plasma membrane, which is essential for the control of ion transport and cytoplasmic pH (for review, see Palmgren, 1998). The activity of the H<sup>+</sup>-ATPase is deregulated by the fungal toxin FC, resulting in membrane hyperpolarization and alteration of ionic gradients. This affects a number of plant processes, including cell expansion, seed germination, stomatal behavior, and nutrient uptake (for review, see Marré, 1979). Changes in membrane potential are also associated with the initiation of a number of other signal transduction pathways (for review, see Ward et al., 1995), in particular those involved in pathogen and in stress responses. The first evidence that FC could affect defense signaling was the inhibition of wound-responsive gene expression in tomato (*Lycopersicon esculentum*) (Doherty and Bowles, 1990; O'Donnell, 1994). Subsequently, FC was shown to inhibit systemin-induced depolarization of tomato leaf plasma membranes (Moyen and Johannes, 1996), oligogalacturonic acid-induced expression of Phe ammonia lyase (Messiaen and van Cutsem, 1994), and, more recently, elicitation of active oxygen species in cryptogein-treated tobacco cells (Simon-Plas et al., 1997).

FC binds to a single "receptor" in higher plants, and polypeptides isolated as FC-binding proteins from three

different plant species have been identified as products of the 14-3-3 gene family (Kourthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994). It has now been demonstrated that a functional FC-binding site is formed from a complex between the C-terminal regulatory domain of the H<sup>+</sup>-ATPase and 14-3-3 proteins and that both proteins are required for FC binding (Baunsgaard et al., 1998). However, since another protein-protein complex involving 14-3-3s and the enzyme NR was shown to be disrupted by FC in vitro (Moorhead et al., 1996), the precise mode of interaction between FC and 14-3-3 proteins remains to be established.

14-3-3 proteins are a family of regulatory proteins that have attracted much attention in recent years because of the identification of interactions between various mammalian 14-3-3 isoforms and proteins involved in signal transduction, particularly protein kinases and phosphatases (for review, see Aitken, 1996). Biochemical and structural analyses of 14-3-3 proteins have shown that they occur as homo- and heterodimers in vitro and in vivo, leading to the suggestion that 14-3-3 dimers may mediate interactions between pairs of associated proteins (Jones et al., 1995). It has now been shown that 14-3-3 proteins bind to phosphorylated Ser residues present within one of a small number of consensus sequences found in many of the proteins with which they interact (Muslin et al., 1996; Yaffe et al., 1997; Ku et al., 1998).

A number of 14-3-3 genes have been identified in plants, and functional roles for their products, in addition to their interactions with the H<sup>+</sup>-ATPase, are beginning to be identified. For example, 14-3-3 proteins from Arabidopsis and maize were originally found as part of a transcription factor complex (de Vetten et al., 1992; Lu et al., 1992). It is now known that 14-3-3 proteins complex with the maize transcription factors EmBP1 and VP1 and may function as adapter molecules to establish a complex between the two factors (Schultz et al., 1998). A different role for 14-3-3 proteins is their involvement in the regulation of NR (Bachmann et al., 1996; Moorhead et al., 1996). NR is regulated by phosphorylation, and its activity is inhibited when 14-3-3 proteins are bound specifically to the phosphorylated form of the protein.

We are interested in understanding the regulation of signal transduction pathways leading to responses to pathogens and stress. The aim of this study was to inves-

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Abbreviations: FC, fusicoccin; NR, nitrate reductase; PR, pathogenesis-related; RACE, rapid amplification of cDNA ends.

tigate the effect of FC on PR signaling and to identify tomato 14-3-3 gene products that may be involved. We describe the 14-3-3 gene family of tomato and identify the responses of individual members to FC and PR signals.

## MATERIALS AND METHODS

### Plant Material

Three- to four-week-old tomato (*Lycopersicon esculentum* cv Moneymaker) plants grown in controlled-environment cabinets were used in all experiments, with the exception that cotyledon injections were carried out on 16-d-old seedlings grown under the same conditions. For FC treatments, plants were excised at the base and incubated in 1  $\mu$ M FC for 30 min before being transferred to distilled water. The two youngest fully expanded leaves from each of five plants were harvested and frozen in liquid nitrogen at 0 and 30 min and 1, 2, 4, 6, 8, and 24 h after transfer to water. A control incubation of 8 h in distilled water with no FC pretreatment was also performed. For elicitation of resistance responses, leaves or cotyledons were infiltrated with approximately 30  $\mu$ L of intercellular fluid extracts using a 1-mL plastic syringe.

### 14-3-3 cDNA Cloning by PCR

The template for PCR cloning was derived from a cDNA library made from 2-h-wounded tomato leaf tissue in  $\lambda$ -ZAPII (Stratagene) vector. Total phage nucleic acid was prepared from 1 mL of amplified library stock. 3'-RACE-PCR amplifications were carried out using primer 14-3-3A (sequence 3'-CGACTAGTGCITA(T/C)AA(A/G)AA(T/C)GTI(G/A)TIGG-5'; I = inosine) and the M13 universal primer using *Taq* DNA polymerase (GIBCO-BRL). Products were purified from agarose gels using the GeneClean II kit (Bio 101, Vista, CA), digested with *Spe*I plus *Xho*I, and cloned into pBluescript SK<sup>-</sup> (Stratagene). 5'-RACE was carried out using primer 14-3-3B (sequence 5'-CGACTAGT(G/A)TA(G/A)TC(G/C/A)CC(C/T)TTCAT(C/T)TT-3') and the M13 reverse primer. Gel-purified products were cloned as *Spe*I/*Bam*HI fragments into pBluescript SK<sup>+</sup> (Stratagene).

### Analysis of DNA and Protein Sequence and Structure

Sequencing of single-stranded DNA templates was performed using Sequenase (version 2.0, Amersham), and sequences were compiled and analyzed using the GCG (Genetics Computer Group, Madison, WI) package (version 8.0). Protein-structure modeling was performed using the program Quanta (Molecular Simulations, Burlington, MA).

### DNA and RNA Analysis

DNA was extracted from tomato leaves using a cetyltriethylammonium bromide extraction protocol, as described by Draper and Scott (1988). RNA was extracted from harvested tissue using a scaled-up version of the method of Verwoerd et al. (1989). Nucleic acids were sep-

arated on agarose gels before transfer onto Magna nylon membranes (Micron Separations, Westborough, MA). Probes were prepared by random-primed labeling and were purified on Sephadex G-50 columns before overnight hybridization with filters in 0.25 M sodium phosphate, pH 7.0, and 7% (w/v) SDS at 65°C. Filters were washed in 0.02 M sodium phosphate, pH 7.0, and 1% (w/v) SDS at 65°C. Bands were visualized by autoradiography.

## RESULTS

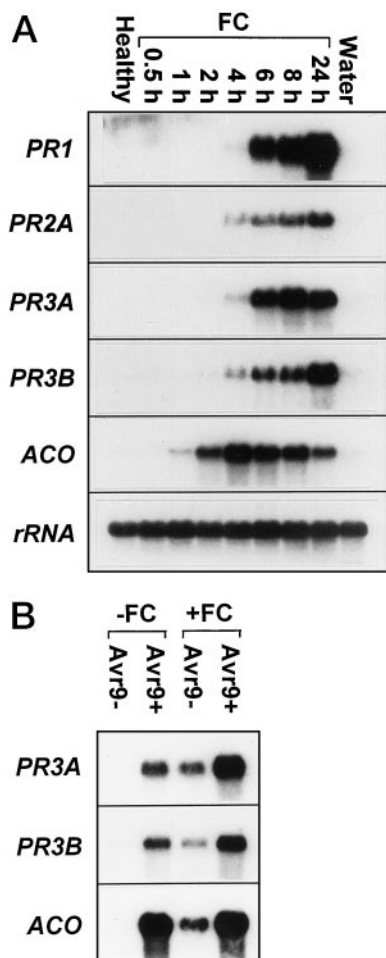
### The Effect of FC on PR Gene Expression

A range of evidence shows that depolarization of the plasma membrane is associated with responses to pathogen elicitors, so we wanted to analyze the effect of hyperpolarizing the membrane with FC prior to challenge with elicitors. We found that FC treatment alone was sufficient to induce PR gene expression. As shown in Figure 1A, a 30-min application of 1  $\mu$ M FC via the transpiration stream of tomato plants led to a rapid, strong induction of mRNAs corresponding to various PR genes. The expression of several classical PR genes, including the basic and acidic isoforms of  $\beta$ -1,3-glucanase and chitinase, was up-regulated after FC treatment. Steady-state message levels of the genes tested increased to high levels from an undetectable baseline within 6 h, with *PR1* and acidic chitinase being particularly abundant. Expression of *ACO* (1-Aminocyclopropane-1-Carboxylic acid Oxidase), which encodes the final enzyme in the biosynthetic pathway of ethylene, was also induced by FC but more rapidly than the PR genes (Fig. 1A). Similar data were obtained for FC-treated tobacco leaves (data not shown).

To investigate the effects of FC on a resistance (R) gene response, we used the well-characterized response induced by the *Cladosporium fulvum* race-specific elicitor Avr9 in tomato plants carrying the *Cf9* R gene (for review, see Hammond-Kosack and Jones, 1995). Co-infiltration of 1  $\mu$ M FC with Avr9-containing intercellular fluids into cotyledons of *Cf9* seedlings produced no visible effect on hypersensitive lesion formation. However, at the level of gene expression, a greater accumulation of the acidic and basic chitinases was observed in the co-infiltrated leaves (Fig. 1B). The co-infiltration also caused a severe downward curvature of the cotyledon petioles that was absent after treatment with Avr9 or FC alone. This resembled a typical epinastic response, which suggests increased ethylene production in the co-infiltrated cotyledons.

### Tomato Contains at Least 10 Members of the FC-Binding 14-3-3 Protein Family

Since FC perception is mediated by 14-3-3 proteins in plant cells and since in one system genes encoding 14-3-3 proteins have been shown to be up-regulated in response to a pathogen (Brandt et al., 1992; Andersen, 1997), we were interested in analyzing the 14-3-3 genes of tomato, with a view to understanding their role in defense responses. Tomato 14-3-3 cDNAs were cloned from a tomato leaf cDNA library using degenerate 3'-RACE-PCR. We defined



**Figure 1.** Effects of FC on PR gene expression. A, PR gene expression is induced by FC, as shown by RNA gel blots showing expression of *PR1* (accession no. M69248), acidic PR glucanase (*PR2A*, accession no. M80604), acidic PR chitinase (*PR3A*, accession no. Z15141), basic PR chitinase (*PR3B*, accession no. Z15140), and ACC oxidase (*ACO*, accession no. A35021) in tomato leaves pretreated with either 1  $\mu$ M FC or water for 30 min. Hybridization to a 28S rRNA probe is shown as a loading control. The data are representative of at least three independent experiments. B, Synergistic effects of FC and avirulence elicitor on PR gene expression, as shown by RNA gel blots showing expression of acidic PR chitinase (*PR3A*), basic PR chitinase (*PR3B*), and *ACO* in tomato cotyledons 24 h after injection with combinations of 1  $\mu$ M FC and intercellular fluid extracts containing (*Avr9+*) or lacking (*Avr9-*) the *Avr9* elicitor.

six distinct sequence classes of 14-3-3 cDNAs on this basis. cDNA clones within each group had identical nucleotide sequences but exhibited significant heterogeneity in the lengths of their 3'-untranslated regions, suggesting the presence of multiple sites of polyadenylation of the mRNAs. Two of the six classes had nucleotide sequences identical to two previously reported tomato 14-3-3s expressed primarily in stems, roots, and fruits (Laughner et al., 1995).

Alignment of the nucleotide sequences of the 3'-RACE products enabled a second degenerate oligonucleotide primer to be designed for 5'-RACE amplifications. The

overlap between the 3'- and 5'-RACE cDNA clones was used to match corresponding products and to construct full-length cDNA sequences. In addition to the original six cDNAs whose sequences were completed in this way, the 5' regions of four other 14-3-3 cDNAs were identified. Each of the 10 classes of cDNA clones is derived from a unique gene. We have designated these 10 genes *TFT1* to *TFT10* (Tomato Fourteen-Three-three). The genes described here as *TFT3* and *TFT4* correspond to those described by Laughner et al. (1995) as Le GF14 T3 and Le GF14 T4, respectively (accession nos. L29151 and L29150), except that the *TFT3* sequence is full length.

Table I presents a summary indicating the level of amino acid identity between the deduced translation products of the *TFT* genes and, for comparison, between the *TFT* sequences and human 14-3-3 $\tau$ . A dendrogram derived from an alignment of the tomato 14-3-3 sequences, human 14-3-3 $\tau$  and 14-3-3 $\epsilon$ , and the 10 Arabidopsis 14-3-3 gene family members shows that the plant 14-3-3 proteins fall into two major groups. Each group is distinct from 14-3-3 $\tau$ , but one includes 14-3-3 $\epsilon$  (Fig. 2). These groups correspond to those described by Wu et al. (1997). The dendrogram also suggests that there is not an equivalent tomato 14-3-3 protein for each Arabidopsis 14-3-3 protein.

#### Predicted Structural Features of Tomato 14-3-3s

The crystal structures of two mammalian 14-3-3 isoforms ( $\tau$  and  $\zeta$ ) were recently solved (Liu et al., 1995; Xiao et al., 1995). These structures show that 14-3-3 monomers are composed of nine antiparallel  $\alpha$ -helices. Helices 1 to 4 form a dimerization domain, and helices 5 to 9 form a domain involved in target binding. Figure 3A shows the consensus amino acid sequence derived from the six full-length tomato 14-3-3 sequences aligned with the amino acid sequence of human 14-3-3 $\tau$ . Little similarity is seen in helices 1, 2, and 4, which are part of the dimerization domain. However, several blocks of extensive identity that correspond to  $\alpha$ -helices 3, 5, 7, and 9 can clearly be distinguished. These helices are located in the inside of the groove formed by the dimer. Superimposition of the alignment onto the crystal structure for 14-3-3 $\tau$  highlights that throughout the polypeptide the majority of conserved residues are located within the groove, whereas divergent residues are located on the outside of the molecule. This is illustrated for one 14-3-3 monomer in Figure 3B. Since this groove is responsible for binding target peptides (Yaffe et al., 1997), this structural conservation suggests that the tomato 14-3-3 proteins likely have fundamental target-binding properties in common with mammalian and other 14-3-3s.

#### 14-3-3 Genes Exhibit Differential Regulation in Challenged Leaves

To analyze the expression of specific 14-3-3 genes in tomato leaves, we generated gene-specific probes for RNA gel-blot analyses corresponding to 400- to 550-bp regions at the 5' ends of the *TFT* cDNAs. The Southern analysis

**Table 1.** Amino acid identity between *TFT* genes and human 14-3-3 $\tau$ 

Percent identities were calculated using the Gap program (Genetics Computer Group). Only the N-terminal region covered by all tomato sequences was considered in the comparison.

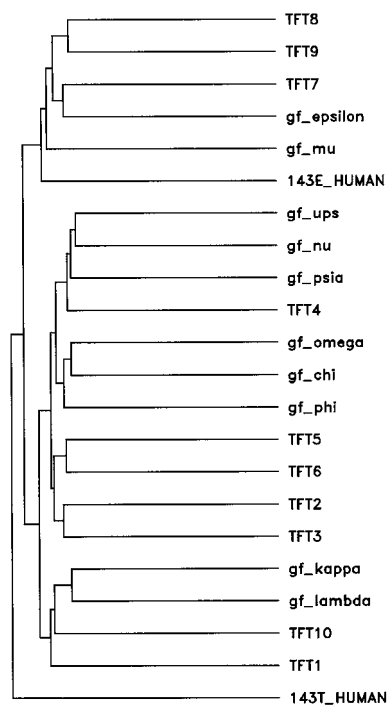
	<i>TFT1</i>	<i>TFT2</i>	<i>TFT3</i>	<i>TFT4</i>	<i>TFT5</i>	<i>TFT6</i>	<i>TFT7</i>	<i>TFT8</i>	<i>TFT9</i>	<i>TFT10</i>
<i>TFT1</i>	–									
<i>TFT2</i>	76	–								
<i>TFT3</i>	72	88	–							
<i>TFT4</i>	66	80	80	–						
<i>TFT5</i>	72	86	85	77	–					
<i>TFT6</i>	72	85	86	77	92	–				
<i>TFT7</i>	57	58	58	65	60	60	–			
<i>TFT8</i>	57	61	58	61	59	60	74	–		
<i>TFT9</i>	60	65	62	69	63	64	74	85	–	
<i>TFT10</i>	78	71	67	71	64	63	57	56	60	–
14-3-3 $\tau$	57	57	53	57	54	53	49	55	54	52

presented in Figure 4 demonstrates that at high stringency each of these probes detects only a single, unique gene.

We first examined expression of the *TFT* genes after treatment of plants with FC. Solutions of 1  $\mu$ M FC were applied to the cut stems of plants for 30 min for uptake via the transpiration stream. Figure 5 shows the expression patterns of the *TFT* genes in leaves over a time course from a representative experiment. Steady-state transcript levels of most of the genes appear relatively unchanged, but significant up-regulation of four genes, *TFT4*, *TFT8*, *TFT9*,

and *TFT10*, was observed repeatedly. The time course of induction of these genes was similar to the induction of PR genes by FC. We then examined the expression of 14-3-3 genes during the *Cf9-Avr9* R gene response and found that most members of the gene family were unresponsive, with no significant change in steady-state transcript level after injection of Avr9 intercellular fluid into *Cf9* leaves.

The results of using *TFT5* and *TFT9* probes are shown in Figure 6 as examples of this lack of response. In contrast, the expression of three genes, *TFT1*, *TFT4*, and *TFT6*, was induced, and the induction was specific to the R gene response since no changes were observed on injection of intercellular fluid into *Cf0* plants, which do not carry the corresponding R gene. Different time courses of induction were observed for each of these three genes. *TFT4* already showed maximum steady-state mRNA levels by the first time point sampled (4 h), and *TFT6* showed a transient accumulation of transcripts to a maximum at 16 h, whereas *TFT1* steady-state mRNA levels showed a gradual increase over the entire time course of 48 h, corresponding closely to the time course of induction of the *PR1* gene used as a control for a positive response (Fig. 6).



**Figure 2.** Relationship among tomato, Arabidopsis, and human 14-3-3 sequences. Dendrogram was produced by the GCG program PileUp and represents amino acid sequence similarity between tomato and Arabidopsis (gf protein) 14-3-3 proteins and human 14-3-3 $\tau$  (Swissprot 143T\_human) and 14-3-3 $\epsilon$  (Swissprot 143E\_human). Only the N-terminal region covered by all tomato sequences was considered in the comparisons.

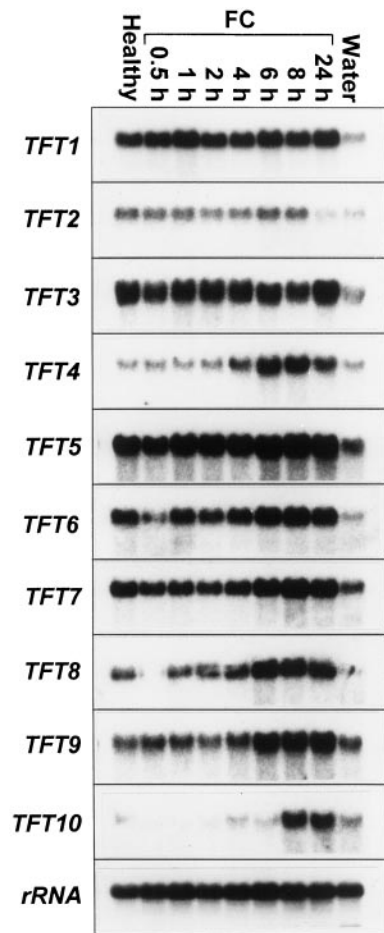
## DISCUSSION

FC has often been used as an experimental tool to investigate the role of plasma membrane depolarization in signaling events leading to plant defense responses. Through its deregulation of the H<sup>+</sup>-ATPase, FC treatment hyperpolarizes the plasma membrane, and this can inhibit downstream responses dependent on membrane depolarization.

It has been shown in tomato that leaf damage or application of oligogalacturonic acids or of the systemin peptide leads to instantaneous membrane depolarization (Thain et al., 1990; Moyon and Johannes, 1996). Pretreatment with FC blocks this depolarization and the effects of wounding and elicitors on the expression of wound-responsive marker genes, such as those encoding proteinase inhibitors (Doherty and Bowles, 1990; Messiaen and van Cutsem, 1994; O'Donnell, 1994). Similarly, the induction of reactive oxygen species by cryptogin, thought in tobacco to act through suppression of H<sup>+</sup>-ATPase activity, is inhibited by







**Figure 5.** Tomato 14-3-3s are differentially expressed in response to FC. Time points showing the mRNA levels in tomato leaves of the 10 tomato 14-3-3 genes through a time course after a 30-min treatment with 1  $\mu$ M FC. RNA from leaves of healthy, untreated plants and from leaves of plants incubated in water for 8 h are included as references. Hybridization to a 28S rRNA probe is shown as a loading control. The data are representative of at least three independent experiments.

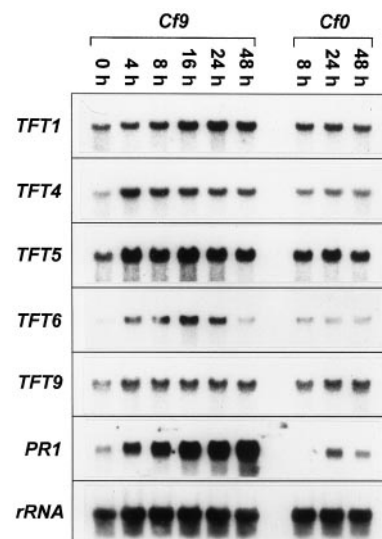
of the 14-3-3 gene family. Initially, it was thought that the 14-3-3 proteins bound FC directly, but recently it has been shown that FC binds only to a protein-protein complex composed of a 14-3-3 protein and the C terminus of the H<sup>+</sup>-ATPase (Baunsgaard et al., 1998). The exclusivity of this target has been questioned by Moorhead et al. (1996), with data showing that FC also affects complex formation between 14-3-3s and NR. However, the effect on NR was demonstrated only in vitro and at FC concentrations much higher than those required for its biological activity.

We have found that tomato plants possess at least 10 14-3-3 genes and that these exhibit differential patterns of expression in the leaf, responding differently to FC and elicitor treatments. These data extend investigations of 14-3-3 gene expression in Arabidopsis and tomato that analyzed tissue-specific expression (Laughner et al., 1995; Daugherty et al., 1996). The question as to whether individual 14-3-3 proteins exhibit functional specificity is currently a matter for debate. In support of this possibility,

some reports indicate a preferential association between particular 14-3-3 proteins and target proteins (e.g. human A20 and 14-3-3 $\beta$  [Vincenz and Dixit, 1996]), whereas *Drosophila melanogaster* 14-3-3 mutants exhibit distinct developmental phenotypes, including lethality for 14-3-3 $\zeta$  (Kockel et al., 1997).

At the protein level there are structural data and a range of biochemical analyses in vitro to argue against a specific role for each 14-3-3 gene product. For example, Yaffe et al. (1997) demonstrated that all of the six human and two yeast 14-3-3 recombinant gene products that they analyzed showed very similar binding characteristics in vitro to phosphopeptide sequences. The major sites of contact with these interacting phosphopeptides are conserved residues found in all mammalian and yeast 14-3-3s and located within the groove formed by a 14-3-3 dimer, as visualized in the crystal structure of the human  $\tau$  protein (Xiao et al., 1995). Any specificity of function of the different 14-3-3 gene products in the cell was suggested to arise via interactions between target proteins and the less-conserved residues on the external face of the dimer (Yaffe et al., 1997). Comparing the tomato 14-3-3 sequences with those of other eukaryotic 14-3-3 genes shows greatest structural conservation within the groove. Thus, it is highly probable that all plant 14-3-3 gene products have the ability to bind the same phosphopeptides in vitro, and this lack of specificity between plant 14-3-3 recombinant proteins and a range of targets has been demonstrated in biochemical studies (Lu et al., 1994; Moorhead et al., 1996; van Heusden et al., 1996; Baunsgaard et al., 1998).

In the plant, functional specificity could be endowed by the cell-specific, inducible, or developmental regulation of 14-3-3 gene expression, such that only a subset of 14-3-3 proteins is present in a cell at any one time. In this context,



**Figure 6.** Tomato 14-3-3s are differentially expressed during a resistance response. Time points showing the mRNA levels in *Cf9* and *Cf0* tomato leaves of selected tomato 14-3-3 genes through a time course after infiltration of Avr9-containing intercellular fluid. Hybridization to a 28S rRNA probe is shown as a loading control. The data are representative of two independent experiments.

transcriptional control of 14-3-3 gene expression has recently been shown to be important in events mediated at the protein level. Thus, transcription of the human 14-3-3 $\sigma$  form was directed by an activated p53 tumor-suppressor protein in response to DNA damage, and the newly synthesized 14-3-3 protein bound to and sequestered Cdc25c, leading to cell-cycle arrest (Hermeking et al., 1997).

It is possible that the responses of the tomato 14-3-3 genes to different stimuli reflect causal roles of their products in the plant. To address this issue we are in the process of producing transgenic lines in which individual 14-3-3 genes are eliminated by antisense mRNA expression. Studies are also in progress to identify targets of 14-3-3 proteins in tomato leaves and to define their interactions during defense responses.

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Accession numbers for the sequences reported in this article are: X95900 (TFT1), X95901 (TFT2), X95902 (TFT3), X95903 (TFT5), X95904 (TFT6), X95905 (TFT7), X98864 (TFT8), X98865 (TFT9), and X98866 (TFT10).

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