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ORIGINAL RESEARCH

Involvement of the Fas-associated Factor1 Ortholog, *CaFAF1*, in Regulating Programmed Cell Death in Plants

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Abstract Programmed cell death (PCD) in plant cells is often accompanied by biochemical and morphological hallmarks similar to those of animal apoptosis. However, orthologs of several core components of such apoptosis have not been reported in plants. Here, we describe an ortholog of Fas-associated factor1 (FAF1), a member of the Fas-death-inducing signaling complex (Fas-DISC), found in pepper. We also examined FAF1 orthologs in other plant species. Transcripts of *CaFAF1* specifically accumulated in pepper leaves infected with the avirulent pepper mild mottle virus (PMMoV) P_0 pathotype. This gene was also strongly expressed in aging leaves. To determine whether those orthologs are involved in PCD, we suppressed their expression through virus-induced gene silencing, and determined the effect on the hypersensitive response (HR), a typical PCD, in pepper, tomato, and tobacco. Constitutive expression of CaFAF1 in transgenic tobacco triggered spontaneous induction of cell death lesions and induced *pathogenesis-related* (*PR*) genes. This ability to cause cell death and suppress *R* gene-mediated HR in its knockdown condition suggests that some features of animal and plant cell death processes may be shared. We propose that plant FAF1 is a conserved cell death regulator in both kingdoms.

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Programmed cell death (PCD) is an essential process for development and an immune response in eukaryotic multicellular organisms. In plants, leaf senescence, the formation of xylem tracheary elements, and host response to pathogens all involve PCD (Lam 2004). Plant cell death occurs during the interactions between plants and pathogens that infect them, although the mechanism and relevant pathway are poorly understood. This PCD can be associated with disease resistance or susceptibility during pathogen attack (del Pozo et al. 2004). A hypersensitive response (HR) occurs rapidly when plants invoke resistance upon an attempted infection (Goodman and Novacky 1994). Although an HR may inhibit further spread of the pathogen, its biological significance has been difficult to verify due to the lack of mutants affected solely by cell death. In disease-susceptible plants, localized cell death, e.g., 'specks', 'spots', or 'blights', can appear over several days. These disease symptoms might also involve host-mediated PCD (Lincoln et al. 2002; Abramovitch and Martin 2004). Thus host-controlled PCD plays a critical role in determining both immunity and disease progression in plants. Despite this significance, relatively few plant genes have been isolated that have a demonstrated role in PCD associated with pathogen attack, and the upstream components that might regulate these cell death mediators have yet to be identified (del Pozo et al. 2004; Greenberg and Yao 2004; Lam 2004).

In *Capsicum* spp., resistance against tobamoviruses conferred by the *L* genes is expressed as a hypersensitive response that results in the induction of necrotic local lesions and a restriction of the virus at the primary infection

sites. It is governed by four seemingly allelic genes (L^1–L^4), which are numbered in order of increasing effectiveness at locus *L* (Boukema 1980, 1982). Correspondingly, tobamoviruses have been classified in terms of increased pathogenicity—pathotypes P_1, $P_{1.2}$, and $P_{1.2.3}$—based on their ability to infect systemically *Capsicum* L^1, L^2, and L^3 resistant plants, respectively (Rast 1979). Elicitation of L^1-mediated resistance requires a functional coat protein from the pepper mild mottle virus (PMMoV) P_0 pathotype (Dardick et al. 1999).

In mammals, PCD has been studied extensively and many proteins involved in this process are known (Deveraux and Reed 1999; Nicholson and Thornberry 2003). PCD, or its morphological trait apoptosis, is genetically regulated cellular suicide. Apoptosis in animal cells is characterized by specific features such as cell shrinkage, blebbing of the plasma membrane, condensation and fragmentation of the nucleus, and internucleosomal cleavage of DNA. The final stage is fragmenting of the cell into cellular debris-containing vesicles called apoptotic bodies, which are then phagocytosed by other cells (Hengartner 2000). The death receptor, Fas, a member of the tumor necrosis factor receptor superfamily, and its ligands are important signaling mediators (Itoh et al. 1991). Apoptosis induced by Fas plays an important role in the development and functioning of the immune system (Opferman 2008). Fas-associated factor1 (FAF1) was originally identified as a Fas-associating molecule with the ability to enhance but not initiate apoptosis when over-expressed in murine L cells (Chu et al. 1995). Later, Ryu et al. (1999) reported that human FAF1 overexpression without any treatment could initiate apoptosis in BOSC23 cells. Recently, hFAF1 has been identified as a member of the Fas death-inducing signaling complex (Ryu et al. 2003) and a suppressor of NF-κB activity (Park et al. 2004). Unlike other Fas-associating proteins, hFAF1 does not contain a death domain but has several homologous domains. Based on amino acid sequence analysis, these include two ubiquitin homologous domains, one UAS domain homologous with *Caenorhabditis elegans* open reading frame C281.1 and another domain homologous with proteins involved in the ubiquitin pathway (UBX) (Buchberger et al. 2001). FAF1 interacts with a subset of additional proteins that are involved in many cellular events, e.g., Fas-mediated apoptosis, heat shock-signaling pathways, and ubiquitin-dependent processes (Kim et al. 2005a).

Here, we isolated an ortholog of *FAF1* from a pepper cDNA library that had been constructed from HR lesions on leaves infected by an avirulent strain, P_0, of PMMoV. Expression of the isolated pepper *FAF1* ortholog was characterized in various organs and in response to PMMoV infection or chemical treatment. To investigate the role of these plant *FAF1* orthologs, we further examined whether *CaFAF1* is expressed in tobacco. Various *FAF1* orthologs were also suppressed in pepper, tomato, *Nicotiana benthamiana*, and tobacco by TRV2-based virus-induced gene silencing (Liu et al. 2002) and their phenotypes were investigated. Our objective was to determine if *CaFAF1* acts as a regulator of PCD.

Materials and Methods

Plant Materials and Growing Conditions

Plants of *N. benthamiana*, pepper (*Capsicum annuum* cv. Bugang), tobacco (*Nicotiana tabacum* 'Samsun NN'), and tomato (*Lycopersicon esculentum* cv. RioGrande-*PtoR* or *PtoS*) were grown in an environmentally controlled glasshouse under a 16-h photoperiod and a temperature range of 20 to 30°C.

Pathogen Inoculation and Chemical Treatments

The bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was grown at 28°C in a King's B medium containing 50 μg mL^{-1} rifampicin. Two strains of pepper mild mottle virus (PMMoV), P_0 and $P_{1.2}$, were propagated on a pepper cultivar and the viral extract was prepared as previously described (Lee et al. 2001). The leaf sap of PMMoV-P_0 or PMMoV-$P_{1.2}$ was prepared by grinding infected leaves in 0.25 M phosphate buffer containing 5 mM EDTA (pH 7.4). To inoculate the approximately 4-week-old plants, virus-containing sap was applied to the surface of four to five fully expanded leaves and rubbed with carborundum (Hayashi Chemical, Osaka, Japan). Mock-inoculated plants were rubbed with phosphate buffer and carborundum only.

Other treatment leaves were sprayed with 1 mM ethephon or 1 mM SA solution while control plants were sprayed only with distilled water. The chemically treated leaves were harvested at 24 h, quickly frozen in liquid nitrogen, and stored at –80°C.

Isolation and Sequence Analysis of *CaFAF1* or *FAF1* Orthologs

A cDNA fragment of *CaFAF1* isolated previously by suppression subtractive hybridization (Shin et al. 2001) was labeled with ^{32}P-dCTP and used as a probe to isolate the corresponding full-length cDNA. Approximately 10^6 plaques from a TMV-induced cDNA library were screened. The resulting positive clones carrying *CaFAF1* cDNAs were excised in vivo from the λZAP expression vector according to the manufacturer's recommendation (Stratagene, USA). Full-length *CaFAF1* cDNA clones were then sequenced from both directions. Orthologs of *FAF1* were isolated from tomato, tobacco, *N. benthamiana*, and rice by PCR using primers

FAF1 homolog for (ATG GBB GAK RYH GYY GAY) and *FAF1* homolog rev (GCC TGM GGR TGY AAW CCW).

CaFAF1 Antibody Production and Immunoblot Analysis

To produce recombinant CaFAF1 protein, a *Bam*HI site of *CaFAF1*, which was the PCR-amplified *CaFAF1* product, was ligated in-frame into the pGEX-KG vector (Pharmacia, UK). The plasmid was transformed into *Escherichia coli* strain BL21. We then performed induction of fusion protein with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and disruption of *E. coli*. GST-fused CaFAF1 protein was solubilized and purified on a GST-chelating column (1 mL, Pharmacia). The purified recombinant protein was injected twice into rabbits for raising polyclonal antiserum.

For immunoblot analyses, 20 ng of GST-fused CaFAF1 and 20 μg each of soluble protein from tobacco, tomato, or pepper leaves were separated on a 12% SDS-polyacrylamide gel and transferred to a nitro-cellulose membrane (Bio-Rad, USA). After blocking overnight in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 80 (TBS-T) containing 5% non-fat dry milk at 4°C, the membranes were incubated with anti-CaFAF1 antibody diluted with TBS-T (1:500) at room temperature for 1 h. After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Promega, USA). The antibody–antigen complex was detected with an enhanced chemiluminescence kit (Amersham, UK). Detection of PMMoV infection via enzyme-linked immunosorbent assay (ELISA) was carried out as described by Lee et al. (2001).

Polyethylene Glycol-Mediated Protoplast Transformation

For analysis of in vivo targeting of CaFAF1, the corresponding cDNA was generated by PCR using primers CJ25 for (5'-AGGATCCCATGAGTTCAACTCAACGAA-3') and CJ25 rev Nonstop (5'-AGGATCCAATGGGCGATGTAGCT GATA-3'). CaFAF1-GFP fusion protein was generated by fusing *CaFAF1* cDNA with the N terminus of the *GFP*-coding region. Constructs were introduced into *Arabidopsis* protoplasts prepared from whole seedlings by the polyethylene glycol-mediated transformation procedure (Jin et al. 2001; Ham et al. 2006). Fluorescence photographs of protoplasts were taken with a Zeiss (Jena, Germany) Axiophot fluorescence microscope fitted with fluorescein isothiocyanate filters (excitation filter, 450 to 490 nm; emission filter, 520 nm; dichroic mirror, 510 nm) and Fuji 400 color film. The optimum exposure time was 1 s.

Generation of Transgenic Plants

The overexpression construct *CaFAF1*-OX was prepared by inserting the full cDNA sequence (digested with *Bam*HI and *Xba*I) into pCAMBIA 2300 containing the *35S* promoter of the cauliflower mosaic virus and the *nos* terminator. Our overexpression construct was introduced into *Agrobacterium tumefaciens* strain LBA4404. pCAMBIA 2300 also was used for transformation and the resulting empty vector-transformed plants served as controls. After sequence verification, *Agrobacterium* carrying the overexpression construct was grown overnight in an AB induction medium (Winans et al. 1988) containing 50 μg ml^{-1} kanamycin and 150 μM acetosyringone. Bacterial cells were harvested and re-suspended in an induction medium to achieve an OD_{600} of 0.1 for transformation. *Agrobacterium*-mediated transformation was performed with newly emerging leaves collected from tobacco cv. Samsun NN, a cultivar that contains the *N* gene. Transgenic plantlets were grown in individual pots in a 1:1 mixture of peat moss/vermiculite. Self-pollinated seeds from independent transgenic lines were harvested. T1 plants carrying *CaFAF1* were selected by germination on MS media containing 50 μg ml^{-1} kanamycin. Positive T1 plants were confirmed by PCR or northern blot analysis by using primers or a probe corresponding to the *35S* promoter and *nos* terminator and/or to the 3' regions of *CaFAF1*.

RT-PCR and RNA Gel Blot Analyses

Total RNA was isolated from the leaves of *N. benthamiana*, tobacco, tomato, and pepper by the hot phenol method (Chomczynski and Sacchi 1987). In all, 20 μg of total RNA from each sample was separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N+, Amersham, USA). The probes were labeled with [α-^{32}P] dCTP using a random-primed DNA-labeling kit and purified on a Sephadex G-25 column (Ambion, USA). Northern hybridization was performed according to standard protocols (Sambrook and Russell 2001). The probe was a 1.1-kb cDNA containing the entire *CaFAF1* open reading frame.

For reverse transcription polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 5 μg of total RNA with MLMV reverse transcriptase (Pharmacia, UK). RT-PCR was performed as described by Park et al. (2001) using the following primers: conserved FAF1 for, CCG CTT ATC GTG CTG CAT TAG AGG and conserved FAF1 rev, CAT CCC ATC GTT CAG CAA GTC GTT (these primer pairs are conserved in all the tested *FAF1* ortholog genes); *NtICS* for, GCT CAT GTG CCG GGT AAA GC and rev, GGA CCA GCA TAC ATT CTT CGG TC; *NtNPR*1 for, GAG GAT ACA TAG GGC ATT GGA TTC T and rev, CTC GAG CTA TTT CCT AAA TGG GAG A; *NtAPX* for, GTTGAAGGTCGCTTGCCTGA and rev, GAAACTTCTTAAGCTGGAAG; *NtHIN1* for, GGT ATG CCT GAA TCG AAC TTG AA and rev, CCC CTA GTC AAG AAT ATC AAC CAT; and *NtSAR8.2* for, TGTTAAT CAAAATGGTTTCCAA and rev, AAAGAGTGCATG

CAGTATCACA. Tobacco *PR-1*, *PR-2*, *PR-3*, *PR-4*, and *PR-5* and *actin* primers were used as described previously (Park et al. 2001; Kim et al. 2005b).

TRV-based VIGS in *N. benthamiana*, *N. tabacum*, *L. esculentum*, and *C. annuum*

Vectors pTRV1 and pTRV2 were kindly given by Dr. Dinesh-Kumar, Yale University, for our gene-silencing trials. A TRV vector for agroinfiltration was constructed as described by Lu et al. (2003). The *CaFAF1*, *NbFAF1*, *NtFAF1*, and *LeFAF1* orthologs were isolated and cloned into a pTRV2 vector using *Eco*RI and *Xho*I to create *TRV::CaFAF1*, *TRV::LeFAF1*, *TRV::NbFAF1*, and *TRV::NtFAF1*, respectively. Infiltration of the approximately 4-week-old plants was performed as described by Liu et al. (2002). *Agrobacterium* strain GV3101 containing pTRV1 or pTRV2 and its derivatives was used for experiments with virus-induced gene silencing (VIGS). The strain was cultured overnight at 28°C in an LB medium. Afterward, the cells were harvested, re-suspended, and diluted in infiltration buffer [10 mM MES, 10 mM $MgCl_2$, and 150 μM acetosyringone (pH 5.6)] to a final OD_{600} of 0.4 (pTRV1) or 0.6 (pTRV2). The resulting mixtures of *Agrobacterium* were incubated for 6 h at 20°C. Plants were infected with TRV and TRV derivatives using 1-ml needleless syringes when the first pair of leaves emerged. After infiltration, plants were kept in a growth room under a photoperiod of 16 h light/8 h dark and a temperature range of 20 to 22°C.

Results and Discussion

Identification of *CaFAF1* from a Pepper cDNA Library

Shin et al. (2001) used differential hybridization analysis to obtain pathogen-induced genes from pepper leaves infected by avirulent strain P_0 of the pepper mild mottle virus. Here, we isolated *CJ25*, a gene induced specifically during the incompatible interaction between hot pepper 'Bugang' and PMMoV. The *CJ25* cDNA is 1,716-bp long and its open reading frame encodes a protein of 468 amino acids (Fig. 1a). The theoretical PI value is 4.99 and its calculated molecular mass is 51.2 kDa.

Pepper *CJ25* is a unique gene; putative plant *CJ25* orthologs have been identified in *Arabidopsis*, rice (*Oryza sativa*), tobacco, *N. benthamiana*, and tomato. The alignment of deduced amino acid sequences for CJ25 showed similarity to unknown *Arabidopsis* proteins containing a UBX domain and a member of the Fas-associated factor1 (FAF1) protein from humans and mice, based on our Blast searches against the NCBI *nr* database (http://blast.ncbi.nlm.gov). The predicted amino acid sequence of the CJ25 protein has two typical domains of UBX (ubiquitin regulatory X) and UAS (a domain of unknown function found in FAF1 proteins). The former is an 80 amino acid residue module that is present typically at the carboxyl terminus of a variety of eukaryotic proteins. Most of the UBX and UAS domain-containing proteins identified so far can be grouped into four evolutionarily conserved protein families, represented by the human Fas-associated factor-1 (FAF1), p47, Y33K, and Rep8 proteins (Buchberger et al. 2001). This pepper putative CJ25 protein also has both conserved domains (Fig. 1a). Our further analysis of plant genome databases available through The Institute for Genomic Research (TIGR) demonstrated that *CJ25* orthologs are likely expressed in other dicots and monocots, many of which are agriculturally important, including tomato, potato, cotton, tobacco, corn, wheat, sorghum, barley, bean, and soybean. Proteins containing regions of domain organization similar to full-length *CJ25* were found via PSI-BLAST (*http://blast.ncbi.nlm.gov*) in humans (GenBank Accession No. NP_008982; 29% identity and 45% similarity), mice (GenBank Accession No. P54731; 28% identity and 46% similarity), and *Drosophila melanogaster* (GenBank Accession No. BAA33466; 27% identity and 43% similarity), suggesting that the function of CJ25 may be conserved between plants and animals. Homologs were also identified in several fungi, such as *Aspergillus nidulans* (GenBank Accession No. XP_658374; 27% identity and 48% similarity), *Ustilago maydis* (GenBank Accession No. XP_758810; 28% identity and 45% similarity), and *Magnaporthe grisea* (GenBank Accession No. XP_366864; 27% identity and 43% similarity) (Fig. 1b). However, a CJ25-like protein was not found in eubacter.

Because the deduced amino acid sequence of *CJ25* shows similarity to a number of unknown proteins and members of Fas-associated factor1 (FAF1) protein from humans and mice, this clone was re-named as *CaFAF1* (GenBank Accession No. AY743432).

Differential Expression of *CaFAF1* During Viral Infection or Senescence

To investigate whether *CaFAF1* is expressed ubiquitously in pepper, we performed RT-PCR and northern blot analysis with total RNA extracted from various organs of healthy and infected plants. A single transcript of 1.7 kb was detected in old leaves and transcript was less abundant in the stem, flower, and fruit. Treatment with ethylene or salicylic acid had no effect on transcript accumulation (Fig. 2a). To evaluate the effect of aging on gene expression, we extracted RNA from the same fourth leaf at different ages. *CaFAF*1 transcripts began to accumulate by 3 weeks after emergence, peaking at week 5, then decreasing slightly (Fig. 2c).

Infection of pepper cv. Bugang, which contains the L^1 resistance gene, induces a localized hypersensitive response

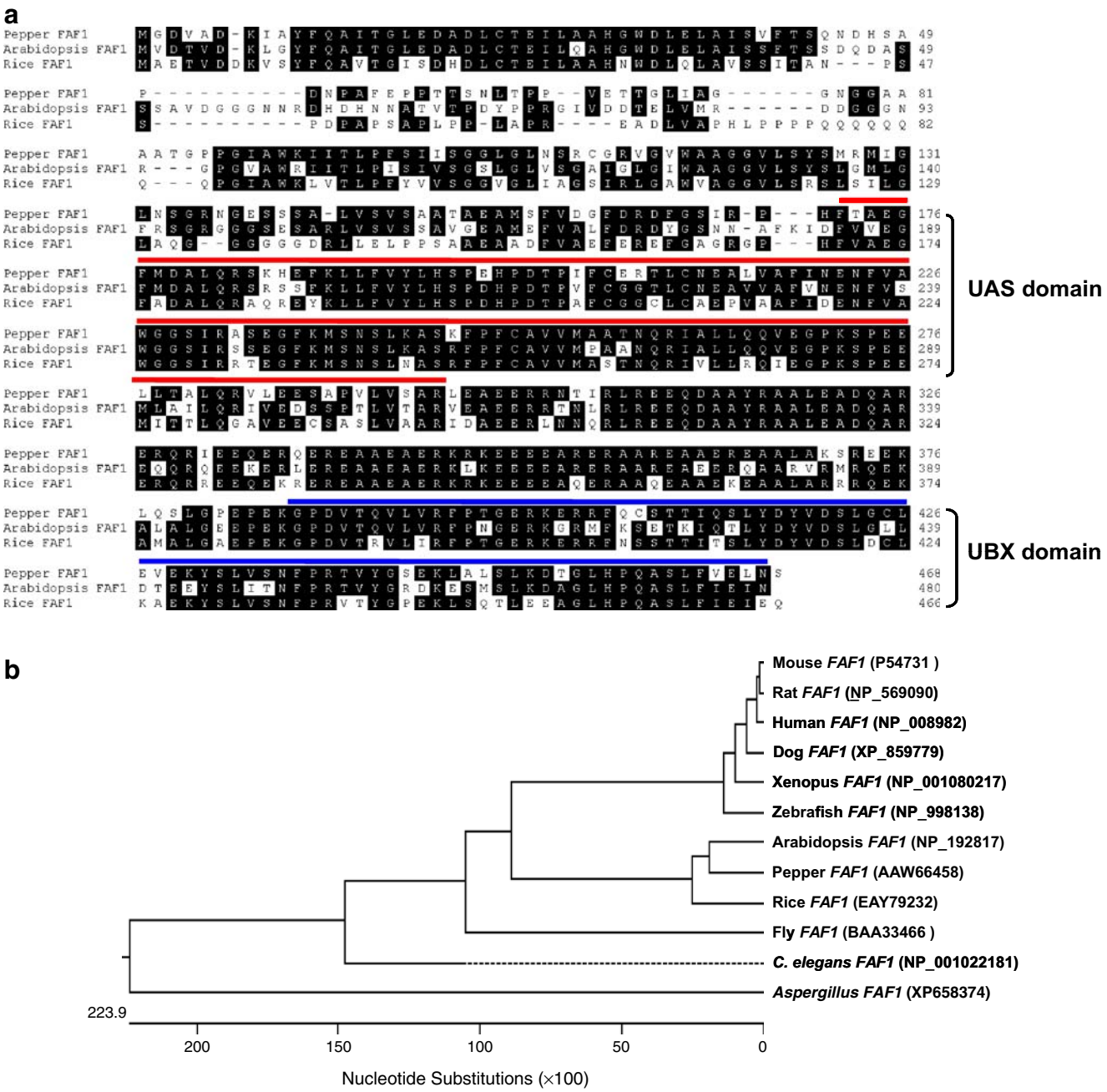

Fig. 1 Comparison of amino acid and nucleotide sequences for pepper CaFAF1 protein. **a** Deduced amino acid sequences of CaFAF1, AtFAF1, and OsFAF1 genes encoding putative FAF1 ortholog proteins that are similar to human or animal FAF1 protein. *Red and blue underlines* indicate positions of two typical domains of UAS (domain of unknown function found in FAF1 protein) and UBX (ubiquitin regulatory X). **b** Phylogenetic analysis of plant *CaFAF1*-like genes. Tree was generated based on nucleotide sequences indexed in GenBank/EMBL BLAST databases. Length of each pair of branches represents distance between sequence pairs; *units* at bottom of tree indicate number of substitution events

with PMMoV, in which lesion development is accompanied by a restriction in viral replication and spread (Shin et al. 2004). Here, a significant accumulation of *CaFAF1* transcripts occurred 24 h after leaves were infected with avirulent strain P_0, but not with the virulent $P_{1.2}$ (Fig. 2b). This suggests that *CaFAF1* is specifically induced during the incompatible response to infection.

To monitor the intracellular localization of CaFAF1 protein, *GFP* fused to the C-terminal region of *CaFAF1* (*CaFAF1-GFP*) was expressed in *Arabidopsis* protoplasts. Vector p2300 with the fusion protein was constructed, prepared, and introduced. Cells expressing that protein displayed a fluorescence localization pattern, as observed in the cytoplasm of those protoplasts (Fig. 3).

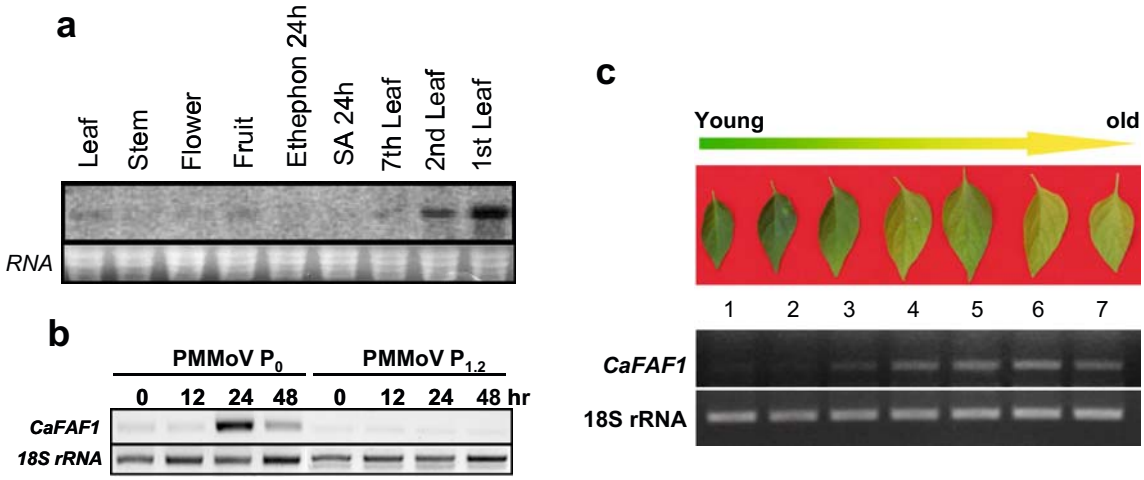

Fig. 2 RT-PCR and RNA gel blot analysis of *CaFAF1* expression in pepper plants. **a** Organ-specific expression. **b** Induction pattern of *CaFAF1* in leaf tissue infected with PMMoV during compatible and incompatible interactions. **c** Induction of *CaFAF1* during leaf senescence. rRNA in agarose gels was stained with ethidium bromide to show equal loading

Silencing of *FAF1* Orthologs Leads to Suppression of *R* Gene-Mediated HR

To investigate the role of *FAF1* orthologs in plants, we used virus-induced gene silencing to knock down the expression of *FAF1* orthologs. VIGS has previously been effective in silencing signaling-related genes in *N. benthamiana*, pepper, and tomato (Ekengren et al. 2003; Chung et al. 2004; Ryu et al. 2004). Each conserved 400-bp fragment corresponding to the 5' region of the *CaFAF1* ORF from pepper, tobacco, and tomato was cloned into a tobacco rattle virus (TRV) silencing vector. VIGS utilizing this fragment resulted in complete silencing of *FAF1* ortholog genes, as shown by RT-PCR analysis of the transcripts and by protein blot analysis of the target protein after pathogen inoculation (Fig. 4b–d). Plants silenced for a *FAF1* ortholog developed normally, exhibiting only slightly delayed senescence (data not shown).

We also examined the response of *CaFAF1*-silenced plants to the PMMoV P_0 strain, which elicits the HR in hot pepper cv. Bugang. This strain was inoculated into the leaves of both *CaFAF1*-silenced and control plants. A hypersensitive response was noted after 24 h in the controls but no cell death was visible on the leaves of *CaFAF1*-silenced plants (left two photos in Fig. 4a). The same results were obtained from three independent experiments.

Because VIGS of *CaFAF1* suppressed the L^1-mediated HR response triggered by the P_0 strain in pepper, we further investigated whether VIGS of *FAF1* orthologs in other plants suppressed the *R* gene-mediated HR. Tomato *FAF1* ortholog *LeFAF1* was silenced to evaluate its role in the *Pto*-mediated resistance response by 'RioGrande'-*PtoR* tomatoes. TRV2::*LeFAF1*-silenced plants were inoculated with *Pst* DC3000, which contains an avirulent protein, AvrPto, that triggers a *Pto*-mediated HR. We observed that resistance was abolished compared with TRV2-only-infected control plants (middle two photos in Fig. 4a).

To quantify the effects of gene silencing of *LeFAF1*, we characterized bacterial growth in two *PtoR*, *PtoS*, and

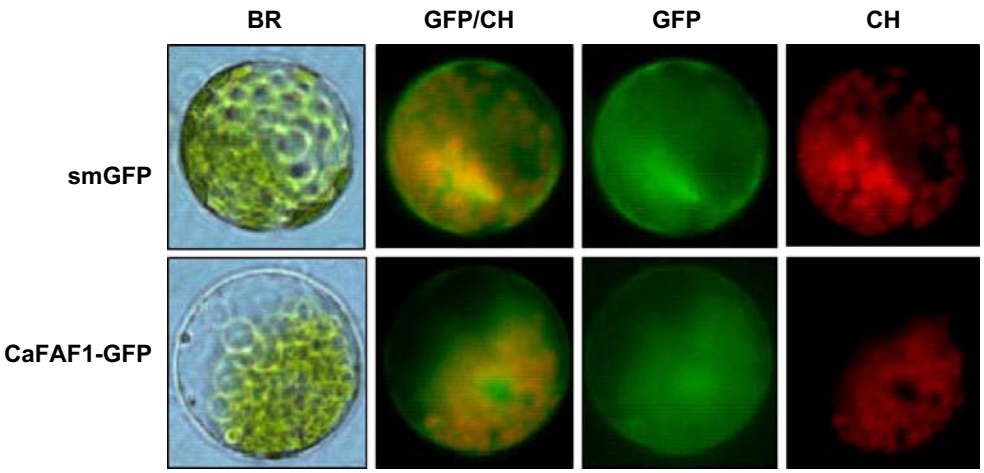

Fig. 3 Subcellular localization of CaFAF1-GFP fusion protein. CaMV *35S*::*GFP*, CaMV *35S*::*CaFAF1-GFP* fusion constructs were independently transformed into *Arabidopsis* protoplasts. Expression of GFP proteins was monitored by fluorescence microscopy under bright and dark fields. *BR* bright-field image; *GFP/CH* overlay of GFP (*green*), and chlorophyll (*red*) images; *SmGFP* soluble modified GFP; *CaFAF1-GFP* CaFAF1 fused in-frame to 5'-end of GFP

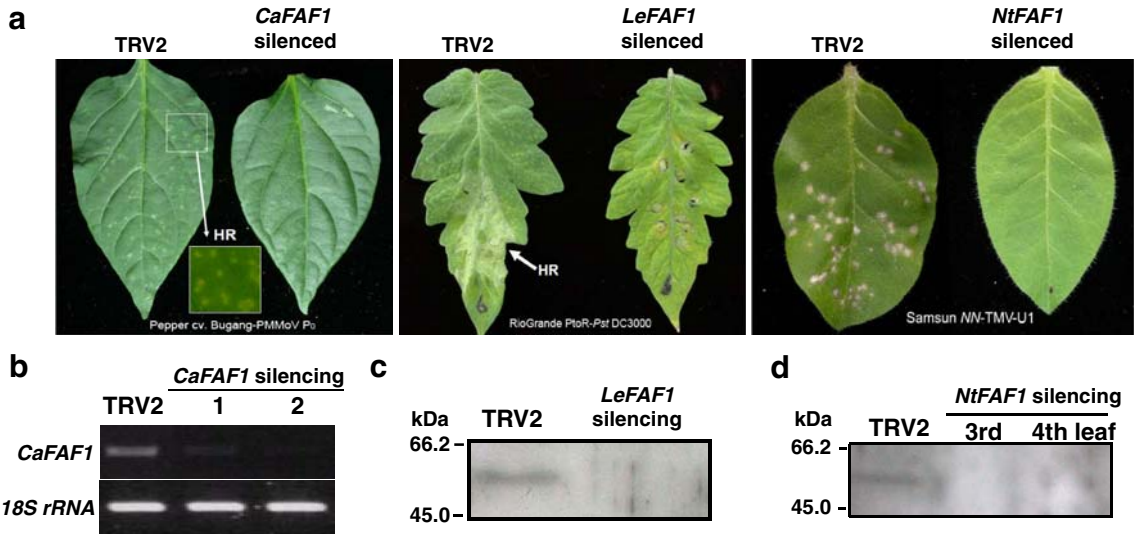

Fig. 4 Phenotypes associated with *CaFAF1, LeFAF1*, and *NtFAF1* gene silencing by TRV2 in plants. **a** Comparison of localized HR induced by PMMoV P_0 strain on pepper leaf, by *P. s*. pv. *tomato* DC3000 on tomato leaf, and TMV-U1 strain on tobacco leaf. **b** RT-PCR analysis of *CaFAF1* expression in empty vector control (TRV2) and *CaFAF1*-silenced pepper leaves (*1*, *2*). *18S rRNA* was used for RNA control. Western blot assay of expression in *LeFAF1*-silenced tomato plants (**c**) and *NtFAF1*-silenced tobacco plants (**d**)

LeFAF1 gene-silenced tomato lines. When plants were inoculated with an avirulent strain, *Pst* DC3000, growth increased approximately fivefold in *LeFAF1*-silenced *PtoR* plants compared with the control *PtoR* (Fig. 5a). However, no differences in bacterial behavior were found between *LeFAF1*-silenced *PtoS* plants and the *PtoS* control, which did not contain the *Pto* resistance gene.

To determine the roles for *NtFAF1* during TMV infection and *N*-mediated resistance, we silenced the endogenous gene and inoculated the TMV-U1 strain to the control and *NtFAF1*-silenced 'Samsun NN' tobacco. Usually, VIGS is not fully effective in all infected leaf cells and tissue areas frequently remain non-silenced. Here, we found that *NtFAF1*-silencing occurred only in the early stage and up to about the fourth leaf. The plants fully recovered later. These results suggest that tissues at the early stage should be used for TRV-mediated gene silencing. In contrast, silencing of *NtFAF1* in 'Samsun' containing the *N* gene completely abolished local lesions that resulted from *N*-mediated HR when compared phenotypically with TRV2 control plants.

Finally we assessed whether *NtFAF1*-silenced plants have altered *N* gene-mediated resistance. TMV was detected via ELISA using a TMV coat protein antibody. The silenced plants exhibited increased accumulation of avirulent TMV in the third or fourth leaves compared with the wild type (Fig. 5b). Eventually, those silenced plants became systemically infected with the viral pathogen.

These experimental results suggested that plant FAF1 proteins act as a positive regulator of host cell death and

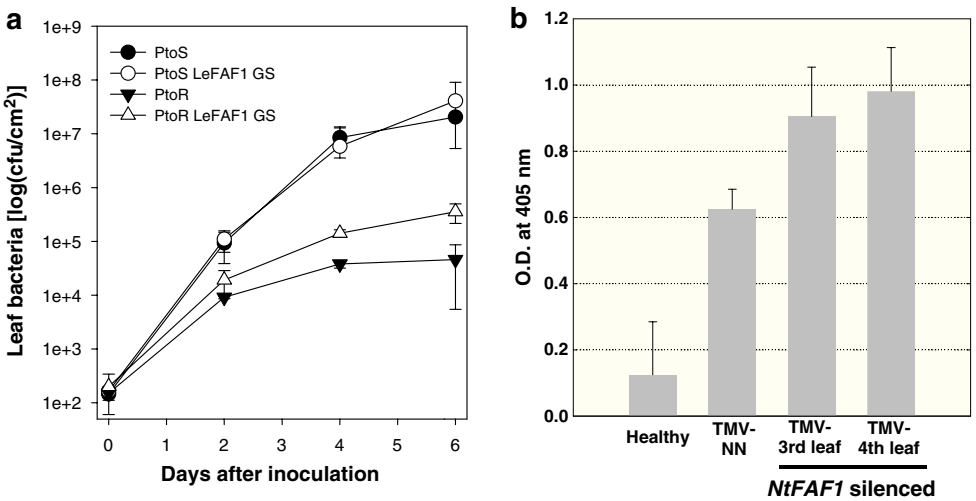

Fig. 5 Decreased resistance by *LeFAF1*-silenced tomato plants to *Pseudomonas syringae* pv. *tomato* DC3000. Leaves of 'RioGrande' *PtoR*, *PtoS*, *LeFAF1*-silenced *PtoR*, and *LeFAF1*-silenced *PtoS* were vacuum-infiltrated with *Pst* suspensions (10^4 cfu ml^{-1}). Bacterial growth in inoculated leaves was determined 0, 2, 4, and 6 days after inoculation (**a**). TMV levels in TRV2-treated 'Samsun NN' and *NtFAF1*-silenced tobacco plants, as determined by ELISA 4 days after virus inoculation (**b**)

that silencing of its orthologs cannot control the hypersensitive response triggered by specific R proteins.

Plant *FAF1* Orthologs Positively Regulate Cell Death Associated with Host Resistance to Viral and Bacterial Pathogens

To study the possible biological functioning of pepper *FAF1* orthologs in plants, we generated transgenic tobacco that constitutively expressed *CaFAF1* under the control of the *CaMV* 35S promoter. Transgenic lines expressing *CaFAF1* were verified by northern blots and RT-PCR analysis. A single signal of approximately 1.7 kb was found in most of those putative transgenics (data not shown), but no signal was detected in control plants. Interestingly, the former often spontaneously formed disease-like necrotic spots on the leaves (Fig. 6a). These lesions appeared first in the oldest, fully expanded leaves while the top three or four (young) leaves never developed them. Lesions may have formed on the mature leaves as they senesced. To determine whether these lesions are HR-like, tobacco leaves were stained with tryphan blue solution. Those from *CaFAF1*-transgenic plants were strongly stained in the necrotic areas while control leaves appeared clean. Our results suggest that the necrotic lesions produced by CaFAF1-expressing tobacco plants resemble the HR-like cell-death lesions found in various disease lesion-mimic mutants of *Arabidopsis*, rice, and maize (Lorrain et al. 2003).

We performed protein blot analysis to determine whether this disease lesion-mimic phenotype is generated by the accumulation of CaFAF1 protein in transgenic tobacco (Fig. 6b). The anti-CaFAF1 antibody detected and showed strong accumulation of that protein in Plants OX1 and 2, whereas tobacco FAF1 protein was detected in wild-type 'Samsun NN'. Thus, it is likely that formation of the lesion-mimic phenotype in the transgenic plants is the result of high-level expression of *CaFAF1*.

Overexpression of *CaFAF1* Activates Expression of *PR* Genes and Causes Pathogen-independent Cell Death on Tobacco Leaves

Plants that produce spontaneous lesions often show elevated expression of genes encoding PR proteins and increased resistance to pathogens (Lorrain et al. 2003). In the absence of such attacks, our *CaFAF1*-transgenic tobacco plants expressed high transcript levels for several *PR* genes that normally are activated during infection. RT-PCR was conducted with 10 gene-specific primers on RNA samples from transgenic and wild-type plants. Fig. 6c shows the relative expression of *PR-1*, *PR-3*, *PR-5*, *SAR8.2*, and *HIN1*, which were constitutively expressed in *CaFAF1*-transgenic plants. This demonstrated the contribution of

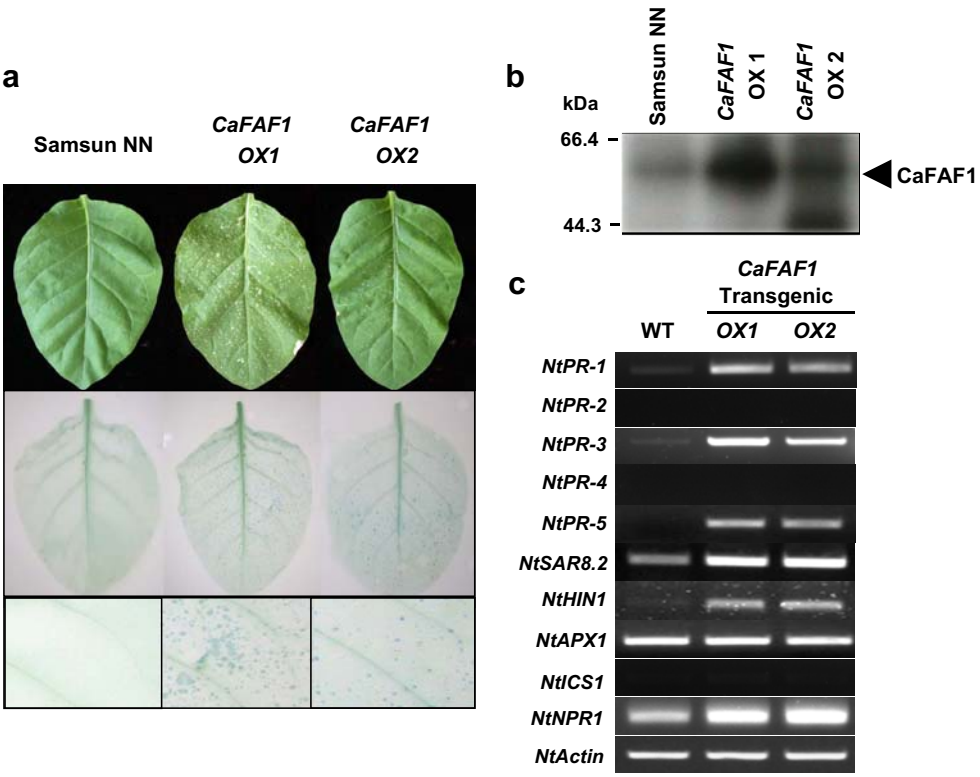

Fig. 6 Phenotypes of transgenic tobacco plants that constitutively expressed *CaFAF1*. **a** Formation of spontaneous lesion-mimic necrotic regions in transgenic *CaFAF1* overexpression lines 1 and 2 (OX1 and 2). Tryphan blue-stained lesion-mimic region in transgenic tobacco (*middle row*) and closer look at spontaneously formed lesions on leaf from transgenic tobacco (*bottom row*). **b** Immunoblot analysis showing elevated CaFAF1 protein level in transgenic tobacco. Total soluble protein was extracted from leaves of wild type and transgenic plants and analyzed for CaFAF1 protein by using anti-CaFAF1 antibody. **c** Expression level analysis via RT-PCR of selected genes, including *PR* genes, in *CaFAF1*-transgenic tobacco

CaFAF1 to the regulation of plant defenses against pathogens. Therefore, we conclude that FAF1 ortholog proteins are able to modulate the cell death response in animal and plants and we can infer that pathways that regulate PCD and defense responses overlap and are conserved, possibly in all higher eukaryotes.

Conclusion

We now provide clear evidence that plant FAF1 functions as a cell death regulator for *R* gene-mediated PCD in various plant species. Thus, plant FAF1 orthologs are good candidates for being conserved regulators. Their presence and functional properties in animal species further suggest that they are cell death regulators, appearing early in the evolution of eukaryotes and preserved in various organisms. We observed increased expression of plant FAF1 during *R* gene-mediated cell death and leaf senescence. Expression of a FAF1 ortholog was suppressed by virus-induced gene silencing, and the effect on the hypersensitive response, a typical PCD in plants, was determined in pepper, tomato, and tobacco. Constitutive expression of *CaFAF1* in transgenic tobacco triggered spontaneous induction of cell death lesions and led to the expression of *PR* genes. These results suggest that CaFAF1 is a component of signal transduction that triggers disease resistance.

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