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Research

Dynamic hydrolase activities precede hypersensitive tissue collapse in tomato seedlings

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Summary

- Hydrolases such as subtilases, vacuolar processing enzymes (VPEs) and the proteasome play important roles during plant programmed cell death (PCD). We investigated hydrolase activities during PCD using activity-based protein profiling (ABPP), which displays the active proteome using probes that react covalently with the active site of proteins.
- We employed tomato (*Solanum lycopersicum*) seedlings undergoing synchronized hypersensitive cell death by co-expressing the avirulence protein Avr4 from *Cladosporium fulvum* and the tomato resistance protein Cf-4. Cell death is blocked in seedlings grown at high temperature and humidity, and is synchronously induced by decreasing temperature and humidity.
- ABPP revealed that VPEs and the proteasome are not differentially active, but that activities of papain-like cysteine proteases and serine hydrolases, including Hsr203 and P69B, increase before hypersensitive tissue collapse, whereas the activity of a carboxypeptidase-like enzyme is reduced. Similar dynamics were observed for these enzymes in the apoplast of tomato challenged with *C. fulvum*. Unexpectedly, these challenged plants also displayed novel isoforms of secreted putative VPEs.
- In the absence of tissue collapse at high humidity, the hydrolase activity profile is already altered completely, demonstrating that changes in hydrolase activities precede hypersensitive tissue collapse.

Introduction

Programmed cell death (PCD) is a highly controlled process that occurs in both plants and animals during development and the immune response. In the context of infection by pathogens, multiple types of PCD have been described in mammals, with apoptosis being the best characterized (Coll *et al.*, 2011). The hypersensitive response (HR) involves a plant-specific type of PCD elicited in the event of pathogen recognition (Heath, 2000). A diverse number of cytological changes are associated with HR, some of which are also shared by animal PCD, examples of which are cytoplasmic shrinkage, chromatin condensation and mitochondrial swelling (Mur *et al.*, 2008). Processes such as vacuolization and chloroplast malfunctioning occur during the last stages of HR and are specific to plant PCD.

The HR is generally, but not always, part of a more complex defence response that leads to resistance to pathogens (Kacprzyk *et al.*, 2011). This form of PCD is often thought to be the ultimate resource to stop invasion of biotrophic pathogens, which depend on living tissue to complete their life cycle. HR is initiated as the consequence of the recognition (either direct or indirect) of the matching pathogen-derived effector by a plant immune receptor. Such an effector is also known as an avirulence (Avr) protein or specific elicitor. Resistance (*R*) genes encode immune receptors and the *R* gene-mediated defence response in plants is often race specific, meaning that the expression of a particular *R* gene confers resistance only against a pathogen species when it harbours the matching effector protein (Romeis *et al.*, 1999; Dodds *et al.*, 2006).

Cladosporium fulvum is a foliar, biotrophic fungal pathogen that colonizes the extracellular space of tomato leaves (de Wit, 1977; Joosten & de Wit, 1999; Thomma *et al.*, 2005). As a result of their co-evolution, tomato has acquired specific R proteins encoded by *Cf* genes, capable of recognizing particular Avr proteins of *C. fulvum*, after which a defence response is activated. Previously, we have reported the development of a simple and robust synchronized system to study HR in tomato, which we have named 'dying seedling' (DS) (de Jong *et al.*, 2002; Gabriëls

et al., 2006; Stulemeijer *et al.*, 2009). DSs are tomato hybrid (F1) plants obtained from a cross between transgenic parental lines (PLs) expressing either the tomato *R* gene *Cf-4* (Hcr9-4D; Thomas *et al.*, 1997) or the corresponding Avr gene from *C. fulvum*, *Avr4*. As the DSs express the *Cf-4*/*Avr4* gene pair in the same tissue, they undergo defence responses which will culminate in the death of the tomato seedling (de Jong *et al.*, 2002). Importantly, activation of HR in these plants is controlled by growth conditions, thereby turning the DS into a versatile biological system to study defence-related hypersensitive cell death. The HR is inhibited when plants are grown at high temperature (33°C) and high relative humidity (RH) (100%) (de Jong *et al.*, 2002; Gabriëls *et al.*, 2006; Stulemeijer *et al.*, 2009). Therefore, at this non-inductive condition, seedlings remain alive and are phenotypically identical to the PLs. In previous studies with DSs, the induction of defence was carried out by transferring the seedlings to a condition of 20°C and 70% humidity. Under these conditions, plants would die within 8 h (de Jong *et al.*, 2002; Gabriëls *et al.*, 2006; Stulemeijer *et al.*, 2009). However, we recently discovered that the transfer from the standard non-inductive condition to a situation in which only the temperature is lowered (from 33 to 20°C) does not induce cell death, as the plants remain phenotypically unaltered for several days (Etalo *et al.*, 2013). It is the successive drop in humidity (from 100% to 70% RH) that ultimately causes plants to die within 90 min (Etalo *et al.*, 2013). In this study, we characterized this synchronized HR further by studying proteome activities using activity-based protein profiling (ABPP).

ABPP displays the active proteome and is based on the use of small-molecule probes that react with the active site of proteins in an activity-dependent manner. Many activity-based probes are specific inhibitors carrying a tag, such as biotin or a fluorescent reporter, to facilitate detection (Cravatt *et al.*, 2008; Kołodziejek & van der Hoorn, 2010). On incubation of the proteome with the probe, only proteins that contain an accessible active site residue will covalently bind to the probe and become irreversibly labelled. ABPP is emerging as a powerful platform to identify the differential activities of proteins acting at the plant–pathogen interface (Kaschani *et al.*, 2009; van der Linde *et al.*, 2012; Lozano-Torres *et al.*, 2012; Shindo *et al.*, 2012).

Here, we used ABPP to investigate the active proteome of tomato seedlings undergoing the HR. Unexpectedly, these assays revealed that the activities of serine hydrolases (SHs) and cysteine protease change before macroscopic cell death occurs. We speculate on the possible effects of temperature and humidity on the activation of Cf-4/Avr4-dependent PCD.

Materials and Methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum* L.) Cf-4/Avr4 DSs were obtained from crossings between Cf-4 (Hcr9-4D)- and Avr4-expressing tomato plants, as described elsewhere (de Jong *et al.*, 2002). Seedlings of the PLs, serving as control, consisted of a 1 : 1 mixture of both genotypes. Sowing and germination of the seeds at 25°C and high RH (100%) were performed as explained in Stulemeijer *et al.* (2009). After 1 wk, the seedlings were transferred to 'non-inductive' conditions (33°C and 100% RH) and, 2 wk later, the temperature was lowered to 20°C. Plant material (cotyledons and primary leaves) corresponding to $t0_1$ was immediately harvested, and plants were kept under these growth conditions (20°C and 100% RH) for three more days. Finally, plants were shifted to the 'inductive' condition by decreasing the humidity to 70% RH. Sampling of additional plant material was performed at the time of decrease of the humidity ($t0_2$) and at 30, 60 and 90 min after the plants had been transferred to 'inductive' conditions. Tissue samples were stored at −80°C until protein extraction. For plant inoculations, the PLs of the tomato DSs, expressing Avr4 (Cf0:*Avr4*; susceptible line) or Cf-4 (Cf0:*Hcr9-4D*; resistant line) were inoculated with *C. fulvum* race 5, expressing Avr4 (de Wit, 1977). At different time points after inoculation (0, 4, 6, 10 and 14 d post-inoculation (dpi)), leaf samples were taken for apoplastic fluid (AF) isolation as described below.

Protein extraction, AF isolation and labelling reactions

Cotyledons and primary leaves were ground to a fine powder with a mortar and pestle using liquid nitrogen. Frozen powder was resuspended in 1 mM dithiothreitol (DTT) to extract proteins. The extract was centrifuged at 16 000 ***g*** for 5 min at 4°C and the supernatant was transferred to a new tube. The protein concentration was measured using the RC/RD protein assay (Bio-Rad) employing bovine serum albumin (BSA) as a standard. AF was isolated from cotyledons and primary leaves of the DSs and PLs, as well as from susceptible and resistant plants inoculated with *C. fulvum* according to Joosten (2012).

For papain-like cysteine protease (PLCP) labelling, 60 μg of total protein extract were incubated either with or without 1 μM DCG-04 (van der Hoorn *et al.*, 2004) in 50 mM sodium acetate (NaAc), pH 6, and 1 mM DTT in a final volume of 0.5 ml. The labelling reaction was performed for 5 h at room temperature (RT) whilst rotating. Proteins were precipitated with 70% acetone and resuspended in 50 μl of 1 × gel loading buffer (GLB). Where indicated, protein samples were pre-incubated for 30 min with 20 μM E-64 before DCG-04 labelling. For the detection of extracellular PLCPs, 100 μl of AF were incubated with 0.2 μM of MV201 in 50 mM NaAc, pH 5.5, and 10 mM DTT in a final volume of 60 μl. After labelling for 3 h at RT, proteins were heated in GLB and separated on protein gels. For inhibition assays, equal volumes of the various AFs were mixed and pre-incubated with 20 μM E-64 for 30 min.

Serine hydrolase (SH) labelling was performed as described by Kaschani *et al.* (2009). Twenty micrograms of total protein or 20 μl of tomato AF were labelled either with or without 1 μM of tri-functional nitrophenolphosphonate (TriNP) or rhodamine-tagged nitrophenolphosphonate (RhNP) (Nickel *et al.*, 2012) in 50 mM Tris, pH 8 (final volume, 50 μl), for 1 h at RT. The labelling reaction was stopped by boiling in 1 × GLB and samples were further analysed as explained later.

For proteasome labelling, 60 μg of total protein were incubated either with or without 2 μM MV151 (Gu *et al.*, 2010) in

50 mM Tris, pH 7, in a final volume of 50 µl. After incubation for 3.5 h at RT, the reaction was stopped by boiling in 1 × GLB. For inhibition assays, equal amounts of all samples were combined and pre-incubated with 50 µM of epoxomicin for 30 min.

The activity of VPEs was detected by incubation of 60 µg of total protein extract or 100 µl of AF with 1 µM AMS101 (Misas-Villamil *et al.*, 2013b) in 50 mM NaAc, pH 5, and 10 mM Tris 2-carboxyethyl phosphine (TCEP) for 2 h at RT. Competition assays were performed by pre-incubation of the protein sample or a mixture of all AFs with 50 µM YVAD-CMK (tyrosyl-valyl-alanyl-aspartyl-chloromethylketone) for 30 min. The labelling reaction was stopped by boiling in 1 × GLB, including an earlier precipitation step with 70% acetone for the AF samples.

Protein samples were separated on 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein gels. Fluorescently labelled proteins were detected by fluorescence scanning of the gel using the Typhoon FLA9000. For DCG-04 labelling reactions, biotinylated proteins were analysed by protein blotting using streptavidin-conjugated horseradish peroxidase (HRP), as described previously (van der Hoorn *et al.*, 2004).

Affinity purification and identification of SHs

Large-scale labelling and purification of biotinylated SHs were performed as described by Kaschani *et al.* (2012). Five milligrams of total proteins were incubated either with or without 200 µM TriNP. After affinity purification, biotinylated proteins were eluted from the beads by boiling in 1 × GLB and resolved on a 12% SDS-PAGE protein gel. The gel was subsequently scanned, photographed and protein bands were automatically excised. In-gel trypsin digestion was performed according to Kaschani *et al.* (2012).

Data acquisition and processing

Peptide mixtures were analysed using a Thermo/Proxeon Easy nLC II mass spectrometer in a two-column configuration (pre-column: 3 × 100 µm, 5 µm C18AQ medium; analytical column: 10 × 75 µm, 3 µm C18AQ) coupled to an LTQ-Velos ion trap (Thermo Scientific, Limburg, Germany). Peptides were separated over a 100-min gradient running from 5% to 25% acetonitrile in water containing 0.1% formic acid. MS/MS spectra were acquired in centroid mode on multiply charged precursors with an *m/z* between 400 and 1600 Da using a Top20 method with active exclusion for 60 s in a window from 0.2 Da below to 1.6 Da above the precursor mass. The resulting RAW files were de-noised (six strongest peaks per 100-Da window) and converted to mgf format using MSConvert from the Proteowizard package (http://www.proteowizard.org/).

Database searching

The sequence database was constructed by combining the ITAG2.3 tomato protein database (34 727 sequences) from the Sol Genomics Network (www.solgenomics.net) supplemented with 1095 common artefact sequences and reversed copies of all sequences as decoys for false discovery rate (FDR) calculation. MS/MS spectra were searched against the described databases using MASCOT 2.3 (www.matrixscience.com). MASCOT searches included a precursor mass tolerance of 0.3 Da, a fragment mass tolerance of 0.4 Da, one permitted C13, one permitted trypsin miscleavage, fixed carbamidomethyl modification on cysteine and variable oxidation of methionine. Reported proteins were identified on the basis of at least two matching peptides exceeding the MASCOT 95% certainty cut-off.

Quantitative reverse transcription-polymerase chain reaction (q-RT-PCR) and gene expression analyses

Cotyledons and primary leaves were ground to a fine powder with a mortar and pestle in liquid nitrogen and frozen powder was used for total RNA isolation following the manufacturer's instructions (RNA extraction kit; Macherey-Nagel, Leiden, the Netherlands). First-strand cDNA was synthesized using Moloney murine leukaemia virus (MMLV) reverse transcriptase and oligo dT (Promega), and was employed as a template for q-RT-PCR using the SensiMix SYBR-HiROX kit (Biolyne, Luckenwalde, Germany). Primer sequences for the different hydrolase-encoding sequences are provided in Supporting Information Table S1. Tomato actin was used as a reference gene (Table S1).

Trypan blue staining

Cotyledons of PLs and DSs, harvested at the indicated time points, were incubated at 100°C for 5 min in a 0.06% solution of trypan blue in lactophenol and ethanol (1 : 2, v/v) (Cai *et al.*, 2001). After an overnight incubation in trypan blue solution at RT, leaves were destained by replacing the staining solution with choral hydrate several times. Finally, samples were rinsed with water and examined using a light microscope.

Results

Dynamics of protease activities in tomato seedlings undergoing synchronized HR

To induce a synchronized HR, 3-wk-old tomato seedlings of PLs and DSs (Fig. 1a) were transferred from non-inductive conditions (33°C and 100% RH) to inductive conditions (20°C and 70% RH) in two consecutive steps (Fig. 1b). First, only the temperature was lowered from 33 to 20°C and then, 3 d later, the RH was lowered from 100% to 70%. We have named these time points $t0_1$ (just before the decrease in temperature) and $t0_2$ (at the time of decrease of the humidity). This final condition (20°C and 70% RH) causes a very rapid and synchronous tissue collapse in the DS within 90 min, whereas the phenotype of the PL remains unaltered (Fig. 1c). Macroscopic cell death is first manifested in the cotyledons, which collapse within the first 30 min after transfer, with the primary leaves collapsing soon after (Fig. 1c). In order to study enzyme activities during this synchronized PCD process, leaf material was harvested from the PLs and DSs at $t0_1$, $t0_2$ and 30, 60 and 90 min after $t0_2$.

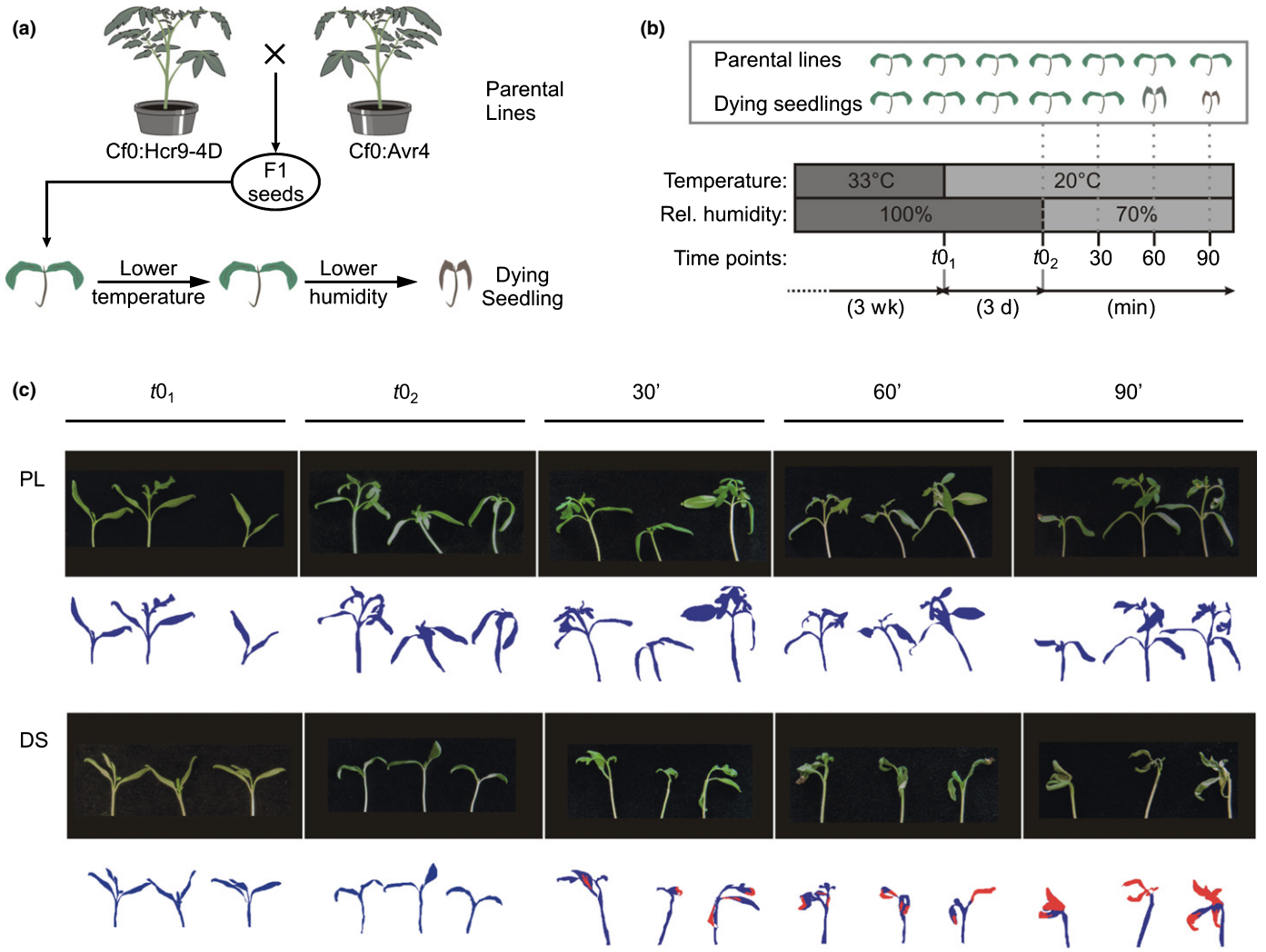


Fig. 1 Experimental design for generating the synchronized hypersensitive response. (a) Tomato (*Solanum lycopersicum*) parental lines (PLs) expressing the resistance gene *Cf-4* (*Hcr9-4D*) or the *Avr4* effector gene from *Cladosporium fulvum* are crossed to obtain F1 hybrid seeds. F1 seedlings are symptomless when grown at elevated temperature (33°C) and 100% relative humidity (RH). Decreasing only the temperature (to 20°C) does not cause any symptoms, but a subsequent drop in humidity causes synchronized hypersensitive cell death, hence the name 'dying seedling' (DS). (b) Two-stage induction of synchronized cell death. Tomato seedlings are grown at 33°C and 100% humidity for 3 wk. At time point $t0_1$, the temperature is decreased to 20°C and seedlings are kept for 3 d at 100% humidity. At time point $t0_2$, the humidity is decreased to 70% and subsequently samples are taken at 30, 60 and 90 min. The DSs are symptomless until $t0_2$ and collapse within 90 min after $t0_2$. The PLs, which do not develop any symptoms on exposure to the same treatment, are taken as controls. (c) Development of tissue collapse in the DS system. Three seedlings of PL and DS were photographed at $t0_1$, $t0_2$ and 30, 60 and 90 min after $t0_2$. To indicate collapsed tissues, the surface area of the healthy and collapsed tissue is depicted in blue and red, respectively.

The activities of the proteasome and vacuolar processing enzymes (VPEs) are differentially regulated during defence signalling (Xia *et al.*, 2004; Hara-Nishimura *et al.*, 2005; Gu *et al.*, 2010; Misas-Villamil *et al.*, 2013b). The proteasome is required for the HR induced in Arabidopsis by avirulent *Pseudomonas syringae* (Hatsugai *et al.*, 2009), whereas VPEs are required for *Tobacco mosaic virus* (TMV)-induced HR in *Nicotiana benthamiana* (Hatsugai *et al.*, 2004). We investigated whether these protease activities were also altered in our DS system. First, extracts from PLs and DSs were prepared and separated on protein gel to show equal protein amounts (Fig. 2a). Next, these extracts were labelled with activity-based probes to display activities of the proteasome and VPEs. Proteasome activities are detected with MV151, a probe which contains vinyl sulfone as a reactive group and a bodipy fluorophore for detection (Verdoes *et al.*, 2006; Gu *et al.*, 2010). Incubation of the proteome of both the PLs and DSs with MV151 resulted in two 25-kDa signals (Fig. 2b). These signals represent the catalytic subunits (β1, β2 and β5) of the proteasome, as pre-incubation with the proteasome selective inhibitor epoxomicin blocked labelling (Supporting Information Fig. S1) (Gu *et al.*, 2010). Surprisingly, labelling was uniform throughout the time course of the experiment, suggesting that no changes in proteasome activity occur on activation of the Cf-4/Avr4-triggered HR.

Labelling of the seedling proteomes with the fluorescent probe AMS101, which targets VPEs (Misas-Villamil *et al.*, 2013b), caused two signals of *c.* 40 kDa (Fig. 2c). The signals have been reported earlier for tomato leaf extracts and probably represent

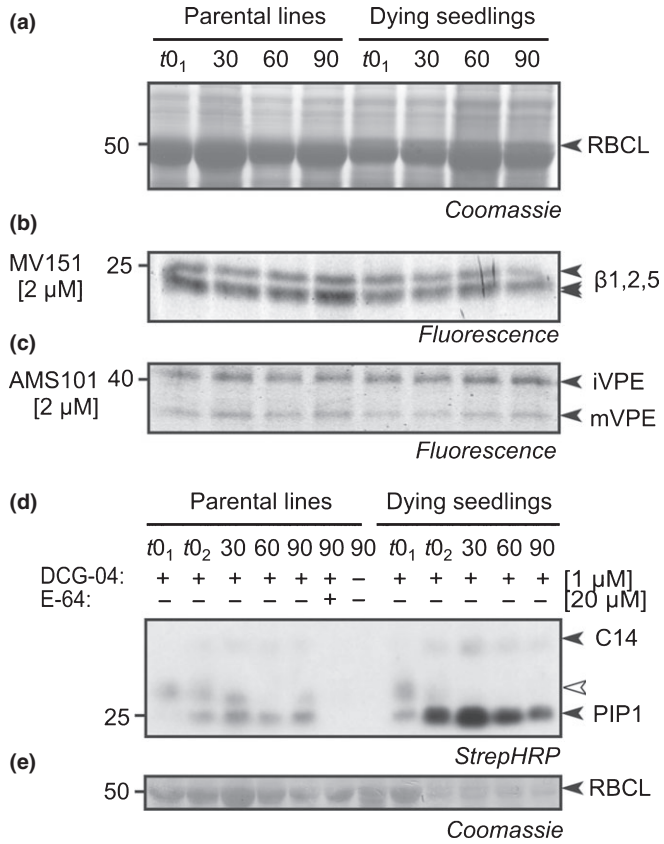


Fig. 2 Tomato (*Solanum lycopersicum*) dying seedlings (DSs) display unaltered vacuolar processing enzyme (VPE) and proteasome activities and increased papain-like cysteine protease (PLCP) activity. Total protein was extracted from parental lines (PLs) or DSs and immediately separated on protein gel (a) or labelled with 2 μM of MV151 (b), 2 μM of AMS101 (c) or 1 μM of DCG-04 (d). After labelling, proteins were separated in protein gels and analysed by Coomassie staining (a, e), fluorescence scanning (b, c) or protein blotting using streptavidin-labelled horseradish peroxidase (HRP) (d). (a) Equal protein levels on extraction in PLs and DSs. RBCL, large subunit of RuBisCo. (b) Activities of the proteasome are not up-regulated in DSs. β1, 2 and 5 refer to the β1, 2 and 5 subunits of the proteasome. (c) Activities of VPEs are unaltered in DSs. iVPE, intermediate VPE; mVPE, mature VPE. (d) Increased activity of PLCPs in DSs. PIP1, *Phytophthora*-inhibited protein 1. (e) Reduced protein accumulation in the DSs during labelling reaction coincides with increased PLCP activity.

both the intermediate (i) and mature (m) isoforms of VPEs (Misas-Villamil *et al.*, 2013b). Labelling of these proteins required a reducing agent and was blocked on pre-incubation with iodoacetamide and the caspase-1 inhibitor YVAD, but not by E-64 (Fig. S2), indicating that the signal represents a cysteine protease that is not from the papain protease class. Labelling is also absent at neutral pH (Fig. S2), known to inactivate VPEs (Kuroyanagi *et al.*, 2002). The sensitivity of labelling for YVAD, which inhibits VPEs (Misas-Villamil *et al.*, 2013b), combined with the selectivity of AMS101 for VPEs by carrying P1 = asparagine (Asn) and P2 = proline (Pro) residues (Misas-Villamil *et al.*, 2013b), indicates that these signals represent VPEs. However, unexpectedly, the labelling pattern of these putative VPEs of the DSs was similar to that of the PLs, and did not change during the time course (Fig. 2c). Taken together, these observations indicate that the activity of neither the proteasome nor VPEs is differentially regulated in the DSs.

We next studied the activities of PLCPs using DCG-04. This activity-based probe is a biotinylated version of E-64, an irreversible inhibitor of PLCPs (Greenbaum *et al.*, 2000), and has been used to display the activities of PLCPs in plants (van der Hoorn *et al.*, 2004; Richau *et al.*, 2012). Labelling of protein extracts from the PLs and DSs with DCG-04 revealed signals of 25, 30 and 35 kDa (Fig. 2d). These signals were not detected on pre-incubation with a saturating amount of the PLCP inhibitor E-64, which also covalently binds to the active site of PLCPs and therefore inhibits labelling with DCG-04. Based on previous studies, we suggest that the 25-kDa signal represents *Phytophthora*-inhibited protein 1 (PIP1) and the 35-kDa signal represents C14 (Tian *et al.*, 2007; Shabab *et al.*, 2008; Van Esse *et al.*, 2008). Importantly, this assay revealed a strong induction of 25-kDa PLCP activity in the DSs, peaking at 30 min (Fig. 2d). The 35-kDa signal followed a similar pattern of induction as the 25-kDa signal, although the activity levels were much lower. Surprisingly, in both PLs and DSs, the 25-kDa signal was already increased at $t0_2$ (20°C and 100% RH) when compared with $t0_1$ (33°C and 100% RH) (Fig. 2d). Therefore, this activity must have been induced on the temperature shift. This observation implies that some of the molecular changes related to HR occur before the decrease in humidity, even though the DSs do not display any symptoms under these conditions.

Unexpectedly, after staining the protein membrane with Coomassie to confirm equal protein loading, we detected a clear reduction in the amount of the large subunit of RuBisCo (RBCL) in the extracts from the DSs (Fig. 2e). This reduction was repeatedly observed in our experiments, despite the fact that equal protein amounts were loaded based on protein concentration measurements and when protein extracts were immediately separated on protein gels (Fig. 2a). Interestingly, the samples showing reduced RBCL levels were the same as those showing increased PLCP activity, namely the DSs at $t0_2$ and the time points thereafter (Fig. 2e). We also noted that RBCL levels were lower in samples that required longer labelling times (data not shown), which suggests that degradation occurred after protein extraction, possibly by increased activities of PLCPs and/or other proteases.

Dynamic changes in the activity of intracellular and extracellular SHs during HR

SHs are another class of hydrolytic enzyme, whose activity has been shown to respond to infection in other plant–pathogen systems (Kaschani *et al.*, 2009). SHs carry an active site serine (Ser) residue and include proteases, lipases, acyltransferases and esterases. To study SHs in the DSs, we used TriNP, a probe containing a phosphonate warhead, a fluorophore for in-gel fluorescence visualization and biotin for affinity purification of labelled proteins (Nickel *et al.*, 2012). Analysis of the activity profile of SHs in total protein extracts of the tomato DSs revealed several signals that were absent in the no-probe control (Fig. 3a). Moreover, the drop in temperature changed the activity profile

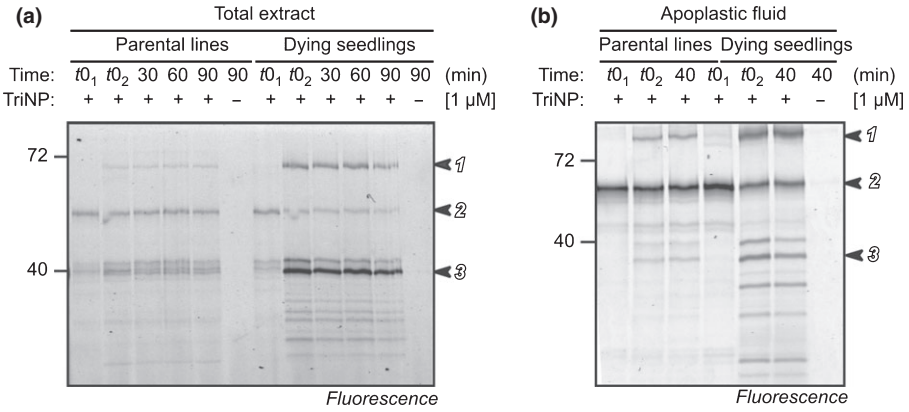


Fig. 3 Tomato (*Solanum lycopersicum*) dying seedlings (DSs) display dynamic alterations in the activities of serine hydrolases (SHs). Total protein (a) and 20 µl of apoplastic fluid (AF) (b), obtained from the parental lines (PLs) and DSs at the indicated time points, were incubated either with or without the trifunctional nitrophosphonate (TriNP) probe. Fluorescently labelled proteins were detected by fluorescence scanning. Major differential signals are indicated by arrows and numbered. (a) Differential SH activities in total protein extracts of the DSs. (b) Differential SH activities in the AF of the DSs.

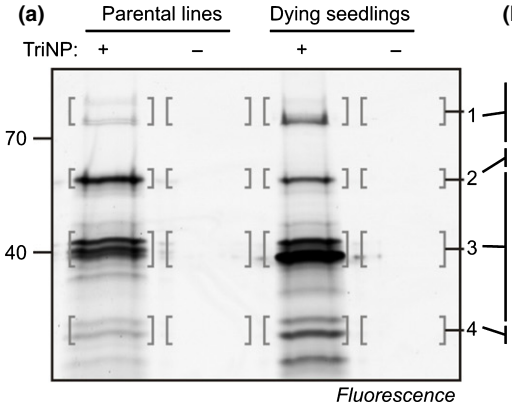
dramatically. A 70-kDa signal (#1) appeared in both PLs and DSs, but was much stronger in DSs (Fig. 3a). A 50-kDa signal (#2), which was present in both PLs and DSs, remained constant in the PLs at the different time points, but was clearly reduced in the DSs on decreasing the temperature ($t0_1$) and remained low on decreasing the humidity (Fig. 3a). Furthermore, a 40-kDa signal (#3) increased on the temperature shift in both PLs and DSs, but was again much stronger in DSs (Fig. 3a). Other low-molecular-weight signals (below 40 kDa) also appeared only in the DS samples at $t0_2$ and later time points (Fig. 3a). The activity profile of the DSs at $t0_1$ was identical to that of the PLs, indicating that there are no major differences in SH activity between PLs and DSs in non-inductive conditions (33°C and 100% RH). These results demonstrate differential SH activity in the DSs.

We next investigated differential SH activities in the apoplast. To this end, we performed a labelling experiment on AF extracted from the PLs and DSs at $t0_1$, $t0_2$ and 40 min after the induction of the HR. This early time point was chosen to avoid leakage from the cytoplasm caused by cell death, which could contaminate the AF with cytoplasmic proteins. Signals #1–3 detected in these AF samples (Fig. 3b) have the same mobility as signals #1–3 detected in the total extracts (Fig. 3b), as shown by running the samples in the same gel (Fig. S3), suggesting that these signals represent the same proteins. Similar to the profiling of total extracts, a 70-kDa signal (#1) increased in intensity at $t0_2$ in the AF of both the PLs and DSs (Fig. 3b). However, this increase was much stronger in the DSs, once again implying a specifically increased activity of this SH in the Cf-4/Avr4-triggered HR. In addition, a 50-kDa signal (#2) decreased in the DSs at $t0_2$, showing the same behaviour as the 50-kDa signal in the total extracts (Fig. 3a). The 40-kDa signal (#3) was reduced in AF relative to the total extracts, suggesting that the most abundant SH causing the signal in the extracts is intracellular. Moreover, several signals, generated by proteins of lower molecular weight, appeared at $t0_2$ in the AF of the DSs (Fig. 3b). To conclude, several differential SH activities detected on the temperature shift are probably secreted proteins.

Identification of differentially active SHs

To identify the enzymes responsible for the differential SH activities, we affinity purified TriNP-labelled proteins and identified them by MS. MS analysis of the purified proteins uncovered 12 SHs belonging to four main protein families (Table S2). Most of these proteins were absent in the no-probe control and many were detected in two independent experiments (Fig. 4a,b). The 70-kDa signal #1 (Figs 3, 4a) contains three S8 subtilisin-like proteases: Solyc08g079870 (P69B), Solyc08g079900 (P69C) and Solyc10g084320 (Fig. 4b). The increased labelling intensity of the 70-kDa signal in the DSs confirms an increased P69 activity during the Cf-4-mediated defence responses. The 50-kDa signal (#2) (Figs 3, 4a) contained the serine carboxypeptidase-like protein (SCPL), Solyc11g066250 (Fig. 4b). The decreased intensity of the 50-kDa signal indicates that the activity of this SCPL is down-regulated during immune responses in the DSs (Figs 3, 4a). In addition, the 40-kDa signal (#3) (Figs 3a, 4a) includes several carboxylesterases (CXEs) (Fig. 4b). The most abundantly detected CXE is the tomato orthologue of the tobacco Hsr203 esterase (Pontier *et al.*, 1994, 1998), indicating that increased Hsr203 activity is causing increased labelling of the 40-kDa signal in the DSs. Finally, in signal #4 (Fig. 4a), we identified an α-hydroxynitrile lyase (AHL, Solyc03g044790) (Fig. 4b). Overall, these results revealed the up-regulated activity of P69 and CXEs dominated by Hsr203, and the down-regulation of an SCPL, preceding tissue collapse of the seedlings undergoing PCD.

We next used real-time RT-PCR to determine the relative transcript levels of the genes encoding the labelled identified proteins (Fig. 4b,c). For that purpose, relative expression levels were determined at 90 min after the humidity drop and at $t0_1$ and $t0_2$. q-RT-PCR analysis revealed that the transcript levels of *P69B* and *P69C* increased progressively in the DSs relative to the PLs, with fold change values of 4.14 at $t0_1$ and 32.74 at $t = 90$ min (Figs 4c, S4). This augmented expression of *P69*s is in accordance with the increased labelling of the 70-kDa signal (#1) in the DSs (Figs 3, 4a). Transcript levels of the SCPL in the DSs were 33 and six times lower than in the PLs at $t0_2$ and $t = 90$ min, respectively, which is



(b)

Accession	Name	*n*	Spectra NP+	Spectra NP–
Solyc08g079870.1.1	P69B	2	861	0
Solyc08g079900.1.1	P69C	1	556	0
Solyc10g084320.1.1	S8	1	39	0
Solyc11g066250.1.1	SCPL/S10	2	2348	5
Solyc02g069800.1.1	HSR203	2	14467	0
Solyc09g075670.1.1	CXE	2	6301	38
Solyc09g075680.1.1	CXE	2	1997	0
Solyc01g108530.2.1	CXE	1	7	0
Solyc01g108580.2.1	CXE	1	253	0
Solyc05g051660.1.1	CXE	1	113	0
Solyc10g054780.1.1	CXE	1	541	0
Solyc03g044790.2.1	AHL	1	34	0

(c)

Relative transcript level DS/PL(t01)	DS/DL(t02)	DS/PL(90')
4.14 ± 1.43	13.83 ± 8.28	32.74 ± 20.9
4.14 ± 1.43	13.83 ± 8.28	32.74 ± 20.9
0.52 ± 0.21	0.86 ± 0.14	1.08 ± 0.13
0.73 ± 0.58	0.03 ± 0.02	0.17 ± 0.04
4.54 ± 0.11	13.50 ± 2.81	7.11 ± 3.10
1.49 ± 0.69	1.81 ± 1.28	6.50 ± 2.47
0.80 ± 0.20	0.97 ± 1.63	1.17 ± 0.18
1.46 ± 0.23	1.20 ± 0.23	2.63 ± 0.33
4.90 ± 0.46	2.35 ± 0.16	13.16 ± 2.31
1.22 ± 0.13	0.94 ± 0.36	1.15 ± 0.01
1.07 ± 0.24	7.56 ± 4.08	8.80 ± 3.99
0.87 ± 0.04	5.57 ± 0.07	30.45 ± 8.07

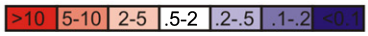


Fig. 4 Differential serine hydrolase (SH) activities are dominated by P69, serine carboxypeptidase-like protein (SCPL) and Hsr203, and are transcriptionally regulated. (a) Identification of labelled SH by MS. Tomato leaf (*Solanum lycopersicum*) extracts from the parental lines (PLs) and dying seedlings (DSs) were harvested at 90 min after decreasing the humidity and incubated either with (+) or without (−) the trifunctional nitrophosphonate (TriNP) probe. Labelled proteins were purified on streptavidin columns and separated on sodium dodecylsulfate (SDS) gels. Fluorescent bands 1–4 were excised from all four lanes and proteins were identified by MS. (b) The experiment was repeated twice and spectral counts for each protein are summed for both experiments (*n*) for the samples labelled with (NP+) or without (NP−) the probe. AHL, α-hydroxynitrile lyase; CXE, carboxylesterase. See Supporting Information Table S2 for more details on protein identification. (c) Fold changes in the transcript levels of the identified SHs at $t0_1$, $t0_2$ and 90 min time points. RNA was isolated at $t0_1$, $t0_2$ and 90 min after decreasing the humidity and used for quantitative reverse transcription-polymerase chain reaction (q-RT-PCR). The DS/PL ratio was calculated for three independent experiments and shown as an average. Errors, ± SD from three experiments. It should be noted that, as a result of the high sequence identity, transcript levels of P69B and P69C were detected with the same primer set and cannot therefore be distinguished from one another. See Supporting Information Fig. S2 for more details on transcript levels.

consistent with the reduced fluorescence signal at 50 kDa (#2; Figs 3a, 4a,b, S4). Of the CXEs detected in the 40-kDa signal (#3), *Hsr203* expression was 13-fold up-regulated at $t0_2$, but less at $t = 90$ min. By contrast, late up-regulated transcript levels were detected for the CXEs Solyc01g108580, Solyc09g075670 and Solyc10g054780, although not as high as for *Hsr203*, whereas transcripts of the three remaining CXEs (Solyc01g108530, Solyc05g051660 and Solyc09g075680) were unaffected. Taken together, the regulation of gene expression seems to underlie the changes in these major protein activities. Interestingly, although both gene expression and protein activity patterns were correlated, the dynamics of these changes were different. When considering transcriptional patterns, the changes occurred either progressively (as in the case of *P69s*) or transiently (for *SCPL* and *Hsr203*). However, SH labelling at these time points did not change accordingly (Figs 3a, S4). Therefore, as changes in transcript levels do not fully correlate with protein activity, P69s, SCPL and CXEs are probably regulated at levels other than just gene expression alone.

Early and late hydrolase activities in the apoplast of infected tomato are responsive to inoculation with *C. fulvum*

To determine whether PLCP, VPE and SH activities also change during infection by pathogens, we studied their hydrolytic activities in the tomato–*C. fulvum* pathosystem. To this end, susceptible (Cf0) and resistant (Cf-4) tomato plants were inoculated with *C. fulvum* producing Avr4. AF was extracted from the inoculated leaves at different time points and the proteins were labelled with probes for PLCPs, VPEs and SHs. As described previously (Joosten & de Wit, 1989), inoculation of both susceptible and resistant plants with *C. fulvum* results in the accumulation of pathogenesis-related (PR) proteins PR2, PR3 and PR7 in the apoplast (Fig. 5).

To monitor PLCP activities during *C. fulvum* challenge, the AF of tomato plants was labelled with MV201, a fluorescent derivative of E-64, which, like DCG-04 (Greenbaum *et al.*, 2000), specifically labels PLCPs (Richau *et al.*, 2012). Labelling showed a 25-kDa signal in the AF of susceptible plants, strongly increasing from 10 to 14 dpi with *C. fulvum* (Fig. 5a). The signal was not detected in the absence of reducing agent, or on pre-incubation of a mix of all AFs with iodoacetamide or E-64, but not with YVAD (Figs 5b, S5), confirming that the signals originate from YVAD-insensitive cysteine proteases. In the AF of resistant plants, this signal appeared earlier (6 dpi) than in susceptible plants, and declined at 14 dpi (Fig. 5a, compare short and long exposure times). The earlier appearance of the 25-kDa signal in resistant relative to susceptible plants resembles the behaviour of PR proteins, whose accumulation increases earlier in incompatible interactions relative to compatible ones (Joosten & de Wit, 1989). It is therefore evident that the defence response activated on recognition of Avr4 by Cf-4 triggers an increase in the extracellular activity of PLCPs. Interestingly, no high PLCP activity was detected in the AF of non-inoculated plants (susceptible and resistant, $t = 0$ dpi, Fig. 5a), suggesting that pathogen challenge induces PLCP activity. Based on its molecular weight and previous MS experiments (Tian *et al.*, 2007; Shabab *et al.*, 2008; Van Esse *et al.*, 2008), we speculate that the majority of the labelled PLCPs represent PIP1 activity. Importantly, the 25-kDa signal intensities resemble the 25-kDa signals observed when labelling total extracts of the DSs (Fig. 2a), confirming that the DS system provides an accurate representation of the events occurring in a tomato plant on challenge with *C. fulvum*. We also detected increased labelling of two bands of *c.* 30 and 35 kDa, which are probably the mature (mC14) and immature (iC14) isoforms of C14, respectively. As for the 25-kDa signal, labelling of 30- and 35-kDa proteins increased in time in both susceptible

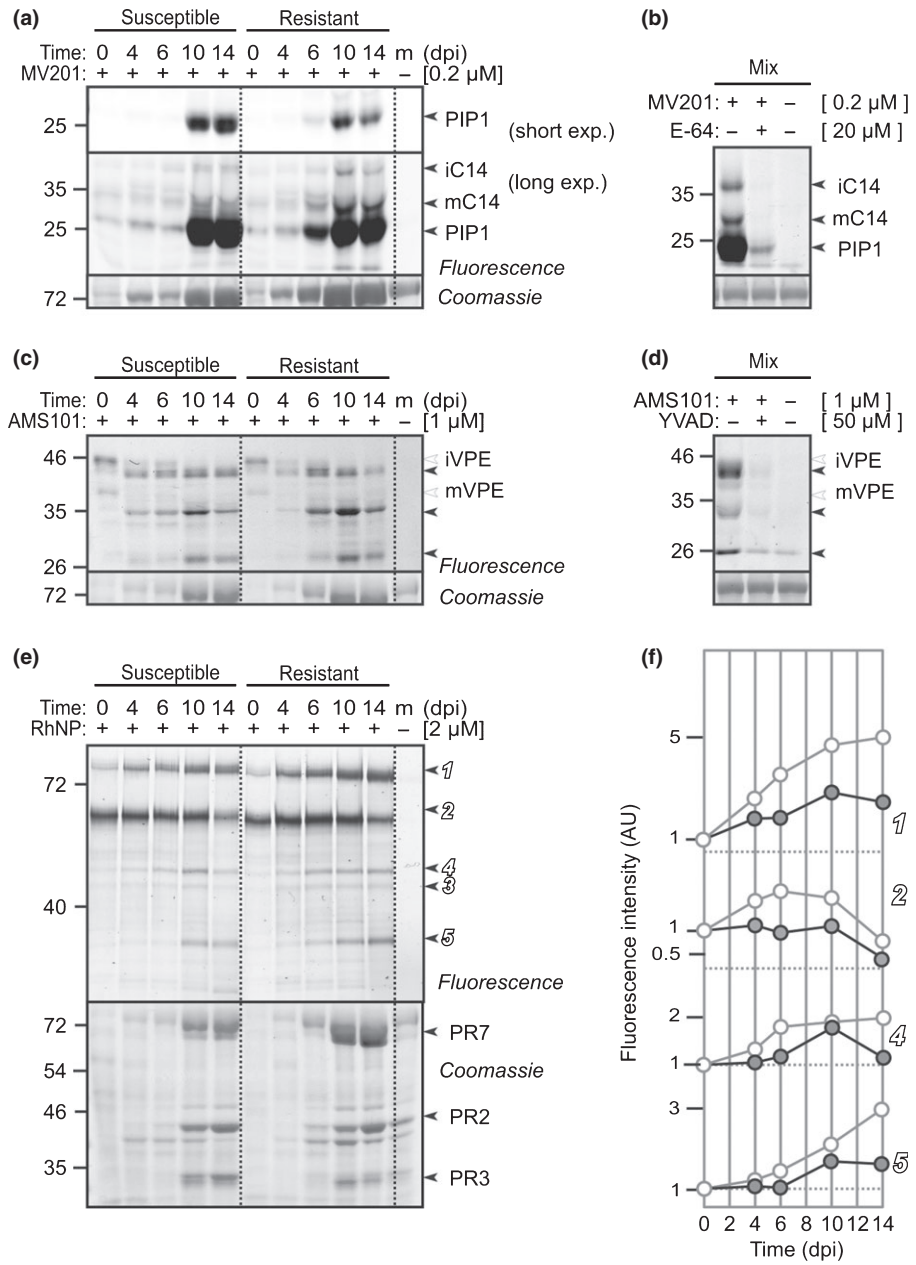


Fig. 5 Challenge of tomato (*Solanum lycopersicum*) plants with *Cladosporium fulvum* induces early and late tomato apoplastic hydrolase activities. (a, b) *Cladosporium fulvum* challenge induces activities of papain-like cysteine proteases (PLCPs). Proteins present in 100 μl of apoplastic fluid (AF) obtained from *C. fulvum*-inoculated susceptible and resistant tomato plants, harvested at 0, 4, 6, 10 and 14 d post-inoculation (dpi), and equal AF volumes, were incubated either with or without MV201 for 3 h. In (b), all AFs obtained from susceptible and resistant plants were combined in equal amounts, pre-incubated either with or without 20 μM E-64 for 30 min and labelled with MV201 as in (a). Labelled proteins were detected by in-gel fluorescence scanning. In (a), m indicates a mix of all AFs. iC14, immature C14; mC14, mature C14; PIP1, *Phytophthora*-inhibited protein 1. The accumulation of PR7 (see Coomassie gel) demonstrates an induced apoplastic immune response. (c, d) *Cladosporium fulvum* challenge alters secreted isoforms of putative vacuolar processing enzymes (VPEs). Proteins present in 100 μl of AF obtained from *C. fulvum*-inoculated susceptible and resistant tomato plants, harvested at 0, 4, 6, 10 and 14 dpi, and equal volumes, were incubated either with or without AMS101 for 2 h. In (d), all AFs were combined in equal volumes, pre-incubated either with or without 50 μM YVAD (inhibitor) for 30 min and labelled with AMS101 as in (c). Labelled proteins were detected by in-gel fluorescence scanning. In (c), 'm' indicates a mix of all AFs. iVPE, intermediate VPE; mVPE, mature VPE. The accumulation of PR7 (see Coomassie gel) demonstrates an induced apoplastic immune response. (e) *Cladosporium fulvum* challenge alters apoplastic serine hydrolase (SH) activities. Proteins present in 20 μl of AF obtained from *C. fulvum*-inoculated susceptible and resistant tomato plants, harvested at 0, 4, 6, 10 and 14 dpi, and equal volumes, were incubated either with or without rhodamine-tagged nitrophenolphosphonate (RhNP) for 1 h. Labelled proteins were detected by in-gel fluorescence scanning. The numbers 1–5 indicate discrete signals that were quantified in (f). m, mix of all AFs. The accumulation of PR2, PR3 and PR7 (see Coomassie gel) demonstrates an induced apoplastic immune response. (f) Quantification of differential SH activities on challenge of susceptible (closed circles) or resistant (open circles) tomato with *C. fulvum*. The intensities of signals 1–5, indicated in (e), were quantified and plotted against time.

and resistant tomato plants. However, this occurred more rapidly and strongly in the resistant plants, once again resembling the behaviour of PR proteins.

The AF of susceptible and resistant tomato plants inoculated with *C. fulvum* was also tested for VPE activity. Interestingly, we detected AMS101 labelling in the apoplast of tomato plants before inoculation ($t = 0$ dpi) with the pathogen (Fig. 5c). These activities probably represent the intermediate (iVPE) and mature (mVPE) isoforms of VPE (Fig. 5c), as labelling is blocked by pre-incubation with iodoacetamide and the caspase-1 inhibitor YVAD, but not by the PLCP inhibitor E-64 (Fig. S6). Labelling did not occur in the absence of reducing agent and at neutral pH (Fig. S6), consistent with the property of VPEs to be active only at acidic pH (Kuroyanagi *et al.*, 2002). This, combined with the high selectivity of AMS101, which carries P1 = Asn and P2 = Pro (Misas-Villamil *et al.*, 2013b), strongly indicates that we are detecting VPE activity in the apoplast of unchallenged plants. Unexpectedly, these signals disappeared and new signals appeared on inoculation with *C. fulvum* of both susceptible and resistant tomato (Fig. 5c). Also in this case, labelling was blocked on pre-incubation with iodoacetamide and YVAD, but not by E-64, and labelling did not occur in the absence of reducing agent or at neutral pH (Figs 5d, S6). These data indicate that VPE-like proteases are present in the apoplast and shift in their mobility on pathogen challenge.

We also performed activity profiling for SHs in the AF of *C. fulvum*-inoculated tomato, using a RhNP probe (Nickel *et al.*, 2012). Four clear signals (#1–#4) were detected in the AF of susceptible plants (Fig. 5e). All signals increased in intensity after inoculation with *C. fulvum* (Fig. 5e), in both susceptible and resistant tomato plants. However, similar to the timing of the increase in PLCP activity (Fig. 5a), the SH activity in the AF of resistant plants accumulated more rapidly and strongly than in susceptible plants. Quantification of the fluorescence intensity confirmed that the intensity of all bands was nearly always higher in the resistant plants than in the susceptible plants (Fig. 5f). Again, this similarity between the two assays indicates that the DS is an accurate biological model system to study immune responses in tomato.

The decrease in temperature is the determining factor triggering differential hydrolase activity

One striking observation throughout our DS experiments was the fact that differences in the SH activity of the PLs and DSs were evident at $t0_2$, which is the time point at which the temperature has been lowered but the humidity is still 100% and tissue collapse is not apparent. In other words, the changes in SH activity appear to precede hypersensitive cell death. To pinpoint more precisely the time at which changes in the SH activity profile occur, we examined the time points between $t0_1$ and $t0_2$ by investigating leaf samples taken at 24 and 48 h after the temperature drop (Fig. 6a). The labelling revealed that changes in the SH activity occurred as rapidly as 24 h after the incubation temperature was decreased from 33 to 20°C in both total extracts and AF (Fig. 6b,c). After another 24 h of incubation at 20°C, additional progressive changes were evident in the labelling profile of AF obtained from the DSs. Most of the differences were specific to the DSs, but the 70-kDa signal was also up-regulated in PLs on the temperature shift, albeit less strongly than in DSs (Fig. 6b,c). The finding that, at 24 h after the temperature drop, massive changes are already visible in the DSs demonstrates that alterations in hydrolase activities precede seedling collapse.

To study when microscopic cell death takes place in our assay, we incubated seedlings in a solution of trypan blue, which stains dead cells blue (Cai *et al.*, 2001), and examined them microscopically. These experiments revealed that, in DSs, some sporadic cell

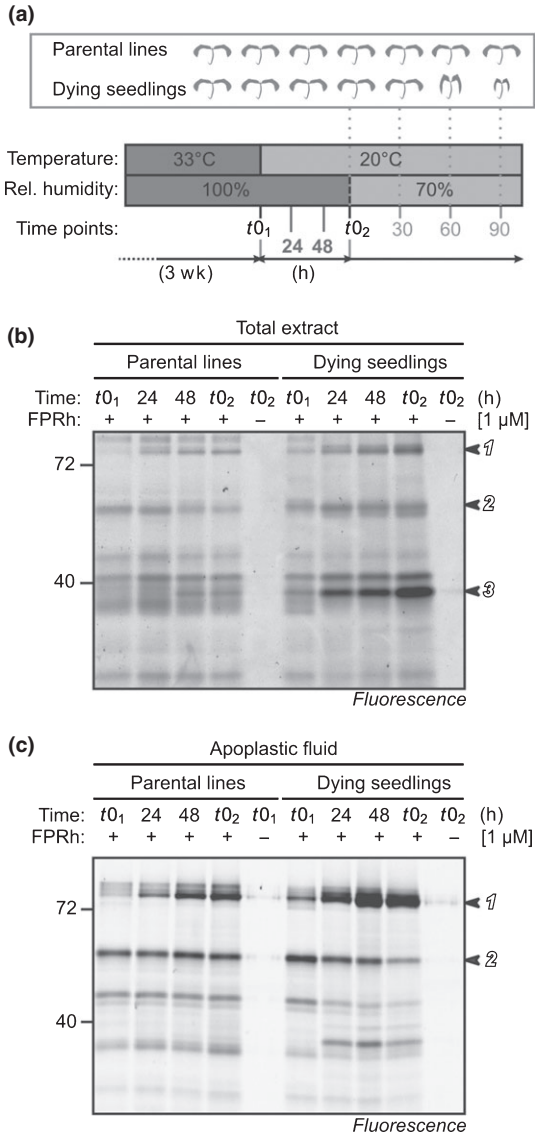


Fig. 6 Differential serine hydrolase (SH) activities in the apoplast precede tissue collapse. Twenty micrograms of protein or 20 μl of apoplastic fluid (AF) were incubated either with or without FPRh (fluorophosphonate-rhodamine) for 1 h at room temperature. Labelled proteins were detected by in-gel fluorescence scanning. (a) Tomato (*Solanum lycopersicum*) seedlings were transferred to the lower temperature at $t0_1$ and samples were taken at 24 and 48 h after transfer. Subsequently, the relative humidity was decreased from 100% to 70% at $t0_2$. (b) Differential SH activities in total extract of the parental lines and dying seedlings on decreasing the temperature. (c) Differential SH activities in the AF obtained from the PLs and DSs on decreasing the temperature.

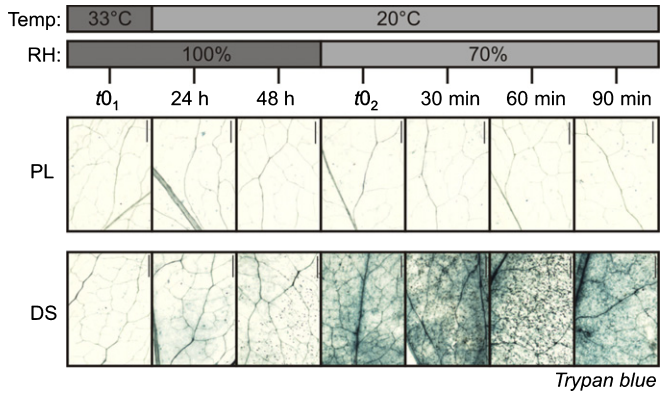


Fig. 7 Decreasing the temperature and relative humidity (RH) induces sporadic and massive microscopic cell death, respectively. Tomato (*Solanum lycopersicum*) parental lines (PLs) and dying seedlings (DSs) were grown as explained in Fig. 6(a), and samples were taken at the indicated time points. To visualize cell death, leaf tissue was stained with trypan blue and microscopic photographs were taken. Photographs are representative of six seedlings. Bars, 500 μm.

death is induced on decreasing the temperature, whereas massive cell death only occurs in these plants on decreasing the humidity (Fig. 7). Thus, despite the absence of macroscopic symptoms (Fig. 1c), DSs at $t0_2$ already show severe cell death (Fig. 7), illustrating that, in this system, PCD is induced very rapidly. These data also show that increased SH activities detected before $t0_2$ coincide with the development of sporadic microscopic cell death, but precede macroscopic hypersensitive cell death and seedling collapse.

Discussion

This study revealed the dynamics of hydrolytic enzyme activities preceding tissue collapse during the HR. We employed a robust biological model system to induce a systemic and synchronized Cf-4/Avr4-triggered HR in tomato, and monitored the activities of VPEs, the proteasome, PLCPs and SHs by ABPP. Our results indicate that changes in the activity of both PLCPs and SHs precede tissue collapse during hypersensitive cell death, and are differentially regulated in the apoplast of leaflets of *C. fulvum*-inoculated susceptible and resistant tomato plants.

VPE activities are unchanged during hypersensitive cell death, but are responsive to pathogen challenge

VPEs have emerged as proteases that regulate PCD in plants (Nakaune *et al.*, 2005). For example, VPE is required for the HR triggered by TMV in tobacco (Hatsugai *et al.*, 2004). However, we did not detect an increase in VPE activity in the DSs on activation of the HR (Fig. 2e). Our finding does not demonstrate that VPEs are required in *Cf4/Avr4*-induced HR; however, our observation is consistent with the finding that the HR is not compromised in a VPE null mutant of Arabidopsis on inoculation with avirulent *Pseudomonas syringae* pv. *tomato* DC3000 (Hatsugai *et al.*, 2009).

Unexpectedly, we detected altered activities of VPE-like proteases in the apoplast of tomato plants inoculated with *C. fulvum*, regardless of whether the plants were susceptible or resistant to this fungus (Fig. 5c). This is remarkable, as VPE activities have so far only been described in the vacuole. Our findings are in contrast with the increased VPE activity detected on infection of Arabidopsis with virulent *Hyaloperonospora arabidopsidis* (Misas-Villamil *et al.*, 2013b), and suggest that the participation of VPEs in defence responses varies in plant–pathogen interactions. Moreover, we observed a conspicuous change in the labelling pattern of putative VPEs in AF of *C. fulvum*-inoculated tomato plants, either susceptible or resistant, when compared with non-inoculated plants (Fig. 5c). The detection of new signals might be the consequence of further processing of these enzymes in the apoplast of tomato leaflets. Alternatively, new VPE-like isoforms might have been induced on inoculation with the fungus, as five VPE genes have been reported to be present in the tomato genome (Ariizumi *et al.*, 2011). In any case, apoplastic VPE-like activity responded to *C. fulvum* challenge, although there was no difference between susceptible and resistant plants. This could reflect an involvement of VPE-like proteases in a basal response to pathogens. Alternatively, these signals may be caused by a secreted fungal protein. However, this is not likely, considering the very small amount of fungal biomass present in resistant plants, especially at later time points. It will be interesting to study the roles and processing of putative VPEs in the apoplast in the future.

Our labelling experiments revealed that the activity of the proteasome is also not differentially regulated during the mounting of the HR in tomato seedlings (Fig. 2d). This is unexpected as the DSs contain large amounts of salicylic acid (SA) (Etalo *et al.*, 2013) and SA is known to activate the proteasome in Arabidopsis (Gu *et al.*, 2010). Furthermore, Hatsugai *et al.* (2009) showed that proteasome activity is required for RPM1-mediated HR in Arabidopsis. In addition, *Pseudomonas syringae* pv. *syringae* produces a proteasome inhibitor that inhibits SA-dependent defence responses and delays hypersensitive cell death (Misas-Villamil *et al.*, 2013b). However, in the case of the DSs, constitutive proteasome activity might be sufficient for the activation of the HR.

Differential activities correlate between assay systems

We showed that PLCP activity is induced before tissue collapse in the DSs (Fig. 2d). Moreover, labelling of the AF of *C. fulvum*-inoculated tomato revealed that PLCP activity increased more rapidly in resistant than in susceptible plants (Fig. 5a). Our data are consistent with previous reports showing the induction of PLCP activity in the apoplast of tomato on benzothiadiazole (BTH, benzothiadiazole; an SA analogue) treatment (Shabab *et al.*, 2008), which was predominantly caused by the accumulation of the PIP1 protease (Tian *et al.*, 2007; Shabab *et al.*, 2008). PIP1 is thought to play a role in plant defence, possibly by degrading effectors and other proteins produced by the pathogen. Our results show that the recognition of fungal Avr4 by tomato Cf-4 increases PLCP activity in the apoplast. The increased PLCP activity is probably associated with increased amounts of PIP1 protein and *PIP1* transcript levels, as described previously (Shabab *et al.*, 2008).

We identified three extracellular subtilisins that belong to the P69 protease family of tomato (Tornero *et al.*, 1996), the activities of which were increased in the DSs and in *C. fulvum*-inoculated tomato (Figs 3, 4, 5e,f). P69 was first identified on inoculation of tomato with citrus exocortis viroid (Granell *et al.*, 1987; Vera & Conejero, 1989). Further studies have shown that some members from this protein family are specifically induced on inoculation with virulent *Pseudomonas syringae* and on treatment with SA (Jordá *et al.*, 1999; Jorda *et al.*, 2000). Little is known about their biological function, although increased expression of P69 proteins on pathogen challenge suggests a role in defence against invading microbes. Subtilisin proteases can have a broad substrate specificity for peptide bonds (Markland & Smith, 1971); therefore, up-regulation of P69 activity might lead to degradation of proteins of the pathogen. Alternatively, subtilases might play a role in signalling. For example, phytaspase is a subtilisin-like protease from tobacco involved in the HR and the resistance response to TMV (Chichkova *et al.*, 2010). Interestingly, phytaspase is also required for PCD induced on abiotic stress (Chichkova *et al.*, 2010).

In addition to P69, we confidently identified several other SHs (Fig. 4a). SCPLs belong to the α/β hydrolase protein family (Breddam, 1986). Although annotated as peptidases, many SCPLs have other enzymatic activities, such as acyltransferase or lyase activity (Li & Steffens, 2000; Mugford *et al.*, 2009). We found that the activity of an extracellular SCPL (Solyc11g066250) was reduced during defence in the DSs (Figs 3, 4a). The expression of its encoding gene was also reduced (Fig. 4b), suggesting that the reduced enzyme activity is a consequence of transcriptional down-regulation.

CXEs comprise another major group of SHs identified here. The activity of an Hsr203-like protein was the most abundantly detected CXE in our assays (Figs 3a, 4a). *Hsr203* was initially identified as a gene specifically induced in the incompatible interaction between tobacco and *Ralstonia solanacearum* (Pontier *et al.*, 1994). Further studies demonstrated that expression of the tomato orthologue, *Slhsr203*, was immediately up-regulated on transient expression of the Avr9 effector from *C. fulvum* in Cf-9-containing tomato plants (Pontier *et al.*, 1998). Since its identification, *Hsr203* transcript levels have been widely used as a hallmark for HR activation (Mishra *et al.*, 2011; Radwan *et al.*, 2011; Zhang *et al.*, 2012). To our knowledge, we are the first to show activity of the Hsr203 protein in the context of a plant–pathogen interaction with a simple and robust labelling assay. We did not detect the activity of Hsr203 in *C. fulvum*-inoculated tomato, either susceptible or resistant, but this is probably because we only studied the AF of the inoculated plants and Hsr203 is not predicted to be secreted.

Differential hydrolase activity precedes hypersensitive tissue collapse

Several of the hydrolytic activities studied in the DSs have been implicated previously in PCD (Pontier *et al.*, 1998). Surprisingly, we showed that changes in the activities of PLCPs and SHs in the DSs precede macroscopic cell death and tissue collapse. Despite the increased PLCP and SH activities, the DSs did not display visible cell death until the humidity was lowered, suggesting that the activation of these hydrolases represents part of a complex immune response. Therefore, although involved in the Cf-4/Avr4-triggered defence response, these hydrolytic activities might not necessarily participate in the signalling cascade leading to hypersensitive cell death itself. Moreover, an elevated temperature was sufficient to repress induced hydrolytic activities of PLCPs and SHs. Wang *et al.* (2005) concluded that both high temperature and 100% humidity synergistically repress Cf-4/Avr4- and Cf-9/Avr9-induced HR. In our experiments, a decrease in the temperature was sufficient to trigger changes in the activity of hydrolases, but not macroscopic cell death.

The temperature-controlled immune response is uncoupled from humidity-controlled hypersensitive cell death

The suppression of hypersensitive cell death by high humidity is not uncommon. Several Arabidopsis lesion mimic mutants show phenotypes that are repressed by high RH (Hammond-Kosack *et al.*, 1996; Jambunathan *et al.*, 2001; Yoshioka *et al.*, 2001; Noutoshi *et al.*, 2005). The *cpr22* Arabidopsis mutant, for example, displays constitutive expression of *PR* genes, spontaneous lesion formation and increased resistance to virulent pathogens, but some of these phenotypes were inhibited at high humidity (Yoshioka *et al.*, 2001). To explain their observations, the authors proposed the existence of a 'humidity-sensitive factor' that down-regulates the SA-dependent defence response at high humidity. The inhibition of effector-triggered PCD by environmental conditions observed in our system could be caused by the crosstalk between biotic and abiotic stress. Specifically, an antagonistic interplay between the ethylene (biotic stress) and ABA (abiotic stress) cascades has been reported previously (Robert-Seilaniantz *et al.*, 2011; Chen *et al.*, 2013). It is tempting to speculate that, when grown at high humidity, the DSs are subjected simultaneously to two different stresses: an ethylene-dependent signalling cascade linked to the HR and an ABA-mediated response linked to the high humidity. In such a situation, hypersensitive cell death would be (partially) repressed by the inhibitory effect of the abiotic signalling pathway. When the humidity is decreased, the pressure of the abiotic stress is released and so is its antagonistic effect on biotic stress signalling. This would then result in the release of the brake on the cell death response. Indeed, a metabolome and transcriptome study on DSs performed by Etalo *et al.* (2013) has indicated that, when the temperature alone is decreased, the production of ethylene is highly induced. However, differential hydrolase activation, which precedes macroscopic hypersensitive cell death, is apparently not under the control of this crosstalk. Moreover, it also suggests that hypersensitive cell death can be uncoupled from other defence responses.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Inhibition of proteasome activity in tomato dying seedlings.

Fig. S2 Characterization of AMS101 labelling of total extracts.

Fig. S3 Comparison of samples on a single protein gel.

Fig. S4 Relative transcript levels of detected serine hydrolases.

Fig. S5 Characterization of MV201 labelling of apoplastic fluids.

Fig. S6 Characterization of AMS101 labelling of apoplastic fluids.

Table S1 Sequences of primers used for quantitative reverse transcription-polymerase chain reaction (q-RT-PCR)

Table S2 Identification of labelled serine hydrolases by MS: information on identified peptides and proteins from two independent experiments

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