Nicotiana tabacum TTG1 contributes to ParA1-induced signalling and cell death in leaf trichomes

Yunpeng Wang[1,*], Ruoxue Liu[1,*], Lei Chen[1,*], Yuancong Wang[1,*], Yuancun Liang[2,*], Xiaojing Wu[1], Baoyan Li[1], Jiandong Wu[1], Yuan Liang[1], Xiaomeng Wang[1], Chunling Zhang[1], Qiuxia Wang[1], Xiaoyue Hong[1] and Hansong Dong[1,‡]

[1]Key Laboratory of Monitoring and Management of Crop Pathogens and Insect Pests, Ministry of Agriculture of P.R. China, Nanjing Agricultural University, Nanjing 210095, China
[2]Department of Plant Pathology, Shandong Agricultural University, Taian 271018, China
*These authors contributed equally to this work
‡Author for correspondence (e-mail: hsdong@njau.edu.cn)

Accepted 5 May 2009
Journal of Cell Science 122, 2673-2685 Published by The Company of Biologists 2009
doi:10.1242/jcs.049023

Summary

Leaf trichomes serve as a physical barrier and can also secrete antimicrobial compounds to protect plants from attacks by insects and pathogens. Besides the use of the physical and chemical mechanisms, leaf trichomes might also support plant responses by communicating the extrinsic cues to plant intrinsic signalling pathways. Here we report a role of leaf trichomes in tobacco (*Nicotiana tabacum*) hypersensitive cell death (HCD) induced by ParA1, an elicitin protein from a plant-pathogenic oomycete. After localized treatment with ParA1, reactive oxygen species were produced first in the leaf trichomes and then in mesophylls. Reactive oxygen species are a group of intracellular signals that are crucial for HCD to develop and for cells to undergo cell death subsequent to chromatin condensation, a hallmark of HCD. These events were impaired when the production of hydrogen peroxide (H_2O_2) was inhibited by catalase or a NADPH-oxidase inhibitor applied to trichomes, suggesting the importance of H_2O_2 in the pathway of HCD signal transduction from the trichomes to mesophylls. This pathway was no longer activated when leaf trichomes were treated with C51S, a ParA1 mutant protein defective in its interaction with *N. tabacum* TTG1 (NtTTG1), which is a trichome protein that binds ParA1, rather than C51S, in vitro and in trichome cells. The ParA1-NtTTG1 interaction and the HCD pathway were also abrogated when *NtTTG1* was silenced in the trichomes. These observations suggest that NtTTG1 plays an essential role in HCD signal transduction from leaf trichomes to mesophylls.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/15/2673/DC1

Key words: NtTTG1, Hypersensitive cell death (HCD), ParA1, Leaf trichome, Signalling

Introduction

Trichomes on plants are epidermal outgrowths of various kinds and are extremely variable in their location on plant organs (Esau, 1965; Uphof and Hummel, 1962). Leaf trichomes are specialized unicellular or multicellular structures derived from differentiation of the epidermal cell layer (Szymanski et al., 2000). The differentiation of leaf epidermal cells and subsequent morphogenesis of leaf trichomes are pivotally controlled by interactions among the TRANSPARENT TESTA GLABRA1 (TTG1) protein, MYB proteins GLABROUS1 (GL1) and TRIPTYCHON (TRY), and the bHLH protein GL3 (Bouyer et al., 2008; Morohashi et al., 2007; Zhao et al., 2008). The TTG1 protein comprises WD40 repeats implicated in protein-protein interaction (Achard et al., 2003) and interacts with the other proteins to promote or inhibit trichome morphogenesis (Morohashi et al., 2007; Zhao et al., 2008). The WD40-MYB-bHLH complex is believed to be an essential regulator of trichome morphogenesis (Walker et al., 1999), and trichome-promoting function of the TTG1-GL1-GL3 complex has been well elucidated in *Arabidopsis thaliana* (Zhao et al., 2008). Inversely, trichome formation is inhibited and epidermal cells remain undifferentiated when TTG1 binds the GL1-TRY complex in the plant (Morohashi et al., 2007). Similar WD40-MYB-bHLH regulatory mechanisms function to control trichome development in tobacco (*Nicotiana tabacum*) and other plants, although leaf trichomes are unicellular in *Arabidopsis* but multicellular in tobacco (Glover et al., 1998). As well as its function in trichome development, TTG1 also affects several other traits, such as biosynthesis of anthocyanin in seeds and cold-treatment-independent termination of seed dormancy (Walker et al., 1999). Therefore, TTG1 is multifunctional in plants to determine the epidermal cell fate and trichome development as well as several other unrelated bioprocesses.

Leaf trichomes play important physiological and ecological roles in plants (Johnson, 1975; Uphof and Hummel, 1962; Wagner, 1991). Trichomes are often implicated in defence responses towards ultraviolet damage and drought stress, reduction of radiation heat load, and the sequestration of toxins (Karabourniotis et al., 1992; Roy et al., 1999; Skaltsa et al., 1994). In many cases, trichomes interfere with the feeding of herbivores (Esau, 1965) and constitute physical defensive structures, which sometimes simply have the potential to impede infestation of insect pests (Ambrósio et al., 2008). Alternatively, plant protection can be provided by natural enemies of insects that impose selection for increased trichome density (Loughner et al., 2008). Evidence also shows the influence of leaf trichomes on foliar fungal infection; to prevent these infections, trichomes are particularly important because they secrete compounds that have antimicrobial activities and contribute to plant defence against pathogens (Amme et al., 2005; Calo et al., 2006; Skaltsa et al., 1994). Thus, depending on circumstances, trichomes can use the physical and biochemical mechanisms to support plant

resistance against insects and pathogens. Plant resistance provided by trichomes via both mechanisms is presumed to decrease fitness owing to the diversion of limiting resources away from present and future growth and development (Mauricio, 1998). This assumption conforms to the requirement for plants to coordinate growth and development with defence responses (Zhang et al., 2007). In the perspective angle of molecular genetics in general, the physical and biochemical roles of leaf trichomes in plant defences are coincident with the activation of distinct signalling pathways (Ambrósio et al., 2008; Amme et al., 2005; Boughton et al., 2005; Kanzaki et al., 2008; Traw and Bergelson, 2003). A central issue in this aspect is how an extrinsic stimulus applied to trichomes is conveyed to a cellular pathway.

In plants, hypersensitive cell death (HCD) associated with a defence response can be induced by a variety of elicitors, such as elicitin and harpin groups of proteins produced by plant pathogenic oomycetes (Kamoun et al., 1993; Liang et al., 2007) and bacteria (Wei et al., 1992; Dong et al., 1999; Dong et al., 2004; Dong et al., 2005), respectively. In response to an elicitor, HCD develops as a microscopic hypersensitive response (micro-HR) with or without subsequent emergence of macroscopic HR (macro-HR). Micro-HR is induced more frequently than macro-HR (Chen et al., 2008; Peng et al., 2004; Takemoto et al., 2005) and is required for macro-HR to be further induced (Peng et al., 2003). Both types of HCD occur as a result of intracellular transduction of reactive oxygen species (ROS) signals, such as hydrogen peroxide (H_2O_2), $O_2^{\bullet-}$, and hydroxyl radicals (Blokhina et al., 2003; Torres et al., 2006; Sagi and Fluhr, 2006). H_2O_2 is an important type of ROS and is often required for HCD to be induced by different elicitors (Alvarez et al., 1998; Quan et al., 2008; Van Breusegem and Dat, 2006).

ParA1, synonym parasiticein (Mouton-Perronnet et al., 1995) or ParaA1 (Baillieul et al., 2003), encoded by the *parA1* gene in *Phytophthora parasitica* var. *nicotianae*, an oomycete pathogen that causes tobacco black shank, is an elicitin protein that can induce plant HCD and resistance to pathogens (Kamoun et al., 1993; Liang et al., 2007). ParA1 belongs to a class of secreted elicitins that are soluble in water and diffusible to plant cells (Baillieul et al., 2003; Huitema et al., 2005; Kamoun et al., 1997). This property provides a basis for the elicitins to be active when externally applied in aqueous solutions to plants by direct treatment of cultured plant cells (Binet et al., 2001; Dorey et al., 1999), by infiltration into leaf intercellular spaces (Costet et al., 2002; Rustérucci et al., 1999), and by diffusion throughout mesophyll cells after immersion of petioles in an elicitin solution (Baillieul et al., 2003). In these cases, elicitin molecules are not directly applied to the insides of plant cells, but instead the molecules get contact with plant cells through diffusion (Yu, 1995). When applied by any of the methods, an aqueous solution of any elicitins can coordinately induce H_2O_2, HCD and other defensive responses in plants (Kanzaki et al., 2008; Liang et al., 2007; Yu, 1995). When an elicitin is applied to plant cells, H_2O_2, cell death and the expression of defence-response genes are induced through the activation of independent pathways (Sasabe et al., 2000). The pathways are believed to activate subsequently to elicitin recognition with particular proteins in plants (Blein et al., 2002; Kanzaki et al., 2008).

We know little about the mechanisms that underlie elicitin perception on the plant-cell surface and subsequent intracellular transduction of the signal. There is also a lack of understanding on whether and how an extrinsic stimulus applied to trichomes is conveyed to a cellular pathway. Our goal in this study is to determine whether live trichomes can transduce ParA1 signal into the cellular HCD pathway and whether a leaf-trichome protein is involved in the process.

Results

ParA1-induced HCD signalling is connected between trichomes and mesophylls

In this study, ParA1 itself, proteins derived from the modification of ParA1 and pertinent control proteins (Fig. 1A) were produced by prokaryotic expression based on the empty and recombinant

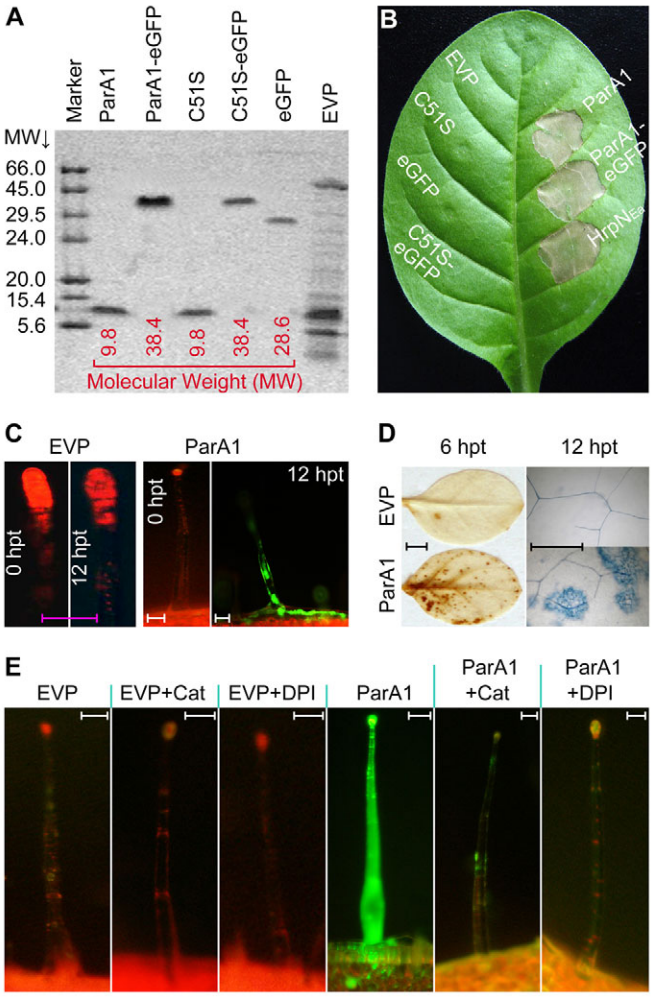

Fig. 1. Characterization and bioassays of the effects of ParA1 and its derivates on leaf trichomes and leaves. (A) Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of the proteins. (B) An assay of the bioactivity in eliciting tobacco macro-HR. $HrpN_{Ea}$, a known HR elicitor used, was used as a control. The image represents 12 leaves photographed at 24 hours post-treatment (hpt). (C) Dichlorofluorescein diacetate (DCFH-DA) fluoluminescence assay of trichomes treated with EVP or ParA1. ROS were shown as green fluorescence in contrast to red background. Each photo represents 60 trichomes. Scale bars: 20 μm. (D) Diaminobenzidine (DAB; left) and Trypan Blue (right) chemiluminescence assays of leaves and mesophylls underneath the trichomes treated with EVP or ParA1. DAB and Trypan Blue stain H_2O_2 and dead cells brown and blue, respectively. Each image represents 12 leaves. Scale bars: 3 cm. (E) DCFH-DA fluoluminescence assay of trichomes treated with the indicated compounds. EVP or ParA1 was applied alone and in combination (+) either with diphenyleneiodonium (DPI), which diminishes H_2O_2 content by inhibiting the activity of NADPH oxidase, or with catalase (Cat), which acts to scavenger H_2O_2. Each image represents 60 trichomes observed at 12 hpt. Scale bars: 20 μm.

vectors (Liang et al., 2007). Because cysteine (Cys) residues in the sequence of ParA1 are crucial for the protein to function (Boissy et al., 1996; Bouaziz et al., 1994), a specific mutant protein, C51S, was created by replacing a Cys with a serine (Ser) at residue 51 in the ParA1 sequence. The resulting ParA1, enhanced green-fluorescence protein (eGFP), and ParA1-eGFP and C51S-eGFP fusion proteins were evaluated in comparison with EVP, the empty vector preparation that contained inactive proteins and was used as a control (Chen et al., 2008; Liang et al., 2007). These proteins were purified by chromatography (Chen et al., 2008) and molecular weights were confirmed by electrophoresis (Fig. 1A). In the bioactivity test, ParA1-eGFP was as effective as ParA1 and the known HCD elicitor $HrpN_{Ea}$ (Peng et al., 2003; Wei et al., 1992) in inducing macro-HR in tobacco leaves, but other proteins were inactive (Fig. 1B).

We observed leaf-trichome responses to ParA1. Surfaces of the top first cells of leaf trichomes were treated with an aqueous solution of ParA1 or EVP by a microinjection-like method (supplementary material Fig. S1). The trichome-growing leaves were excised at 0, 6 and 12 hours post-treatment (hpt) and stained with 2,7-dichlorofluorescein diacetate (DCFH-DA), a red dye that emits green fluorescence when oxidized by H_2O_2 and other types of ROS (Allan and Fluhr, 1997). A strong ROS burst shown as green fluorescence was visualized in ParA1-treated leaf trichomes and untreated mesophylls underneath the trichomes, in contrast to few ROS signs in trichomes at 0 hpt treated with EVP and in ParA1-treated trichomes (Fig. 1C). Apparently, ROS had spread from trichomes to the epidermal and mesophyll cells within 12 hours after trichome treatment with ParA1. By 6 hours after ParA1 application to trichomes, H_2O_2 was detected in the leaves by 3,3-diaminobenzidine (DAB) staining (Fig. 1D), which shows H_2O_2 accumulation visualized as a brown precipitate (Thordal-Christensen et al., 1997). By 12 hpt, micro-HR lesions appeared on leaves (Fig. 1D). These observations indicate a connection of HCD signalling between trichomes and mesophylls.

To confirm the induction of H_2O_2, we conducted pharmacological studies using diphenyleneiodonium (DPI) and catalase. DPI diminishes H_2O_2 content by inhibiting the activity of NADPH oxidase (Balestrasse et al., 2008; Riganti et al., 2004), whereas elicitins trigger a production of H_2O_2 via the NADPH oxidase (Lequeu et al., 2005). Catalase directly scavenges H_2O_2 (Ahn et al., 2007; Zhang et al., 2008). In the leaf-infiltration test, DPI and catalase were inhibitory to the development of ParA1-induced macro-HR on tobacco leaves (supplementary material Fig. S2). When a leaf was infiltrated with a solution containing ParA1 only, complete macro-HR developed in infiltrated areas within 24 hpt. In the same period, however, a merely partial macro-HR was induced in a reduced part of the infiltrated area when catalase or DPI was present in the ParA1 solution, and DPI was greater than catalase in the inhibitory effect. Then, effects of both inhibitors on H_2O_2 were tested in a trichome treatment assay. The use of DPI and catalase caused great decreases in the levels of ParA1-induced ROS in treated trichomes and underlying mesophylls as monitored at 12 hpt. As shown in Fig. 1E, the amount of ROS was much decreased in trichomes treated with ParA1+DPI or ParA1+catalase combinations than those treated with only ParA1. The amount of ROS declined conspicuously but was not completely eliminated when catalase was present in ParA1 treatment compared with control treatment with EVP. In this case, ROS still appeared as a feeble sign. DPI was more effective than catalase to inhibit ParA1-induced ROS production. The effects of both inhibitors were also found in mesophylls. In contrast to the apparent spread of ROS from ParA1-treated trichomes to mesophyll cells, ROS signs evidently decreased in the mesophylls underneath the trichomes treated with ParA1 in the presence of DPI or catalase. Clearly, H_2O_2 occupies a prominent part of the ROS production that is induced by ParA1.

Structural analysis and experimental evidence suggest a ParA1-NtTTG1 interaction

We sought to identify a trichome protein that could interact with ParA1 and contribute to signalling and HCD in leaf trichomes. Initially, we cloned tobacco homologues of *Arabidopsis* genes involved in trichome development, including *GL1*, *GL3* and *TTG1* (*AtTTG1*). Only a homologue of *AtTTG1* responded to ParA1 and this homologue was designated as *NtTTG1* on the basis of further studies. The expression of *NtTTG1* was strongly induced by ParA1 (Fig. 2A,B) in leaf trichomes rather than leaves, suggesting that *NtTTG1* is involved in the ParA1-induced trichome response. The predicted NtTTG1 protein shows a high degree of sequence identity to AtTTG1 (supplementary material Fig. S3). Further experimental evidence suggested that NtTTG1 interacted with ParA1 and functioned in the HCD pathway.

The possibility that NtTTG1 interacted with ParA1 was elucidated by numerous ways. First, three-dimensional structures of TTG1 (Walker et al., 1999; Zhao et al., 2008) and ParA1 (Blein et al., 2002; Boissy et al., 1996; Bouaziz et al., 1994) were characterized by a hydrophobic cavity (Fig. 2C) important to protein-protein interaction. In a yeast two-hybrid (Y2H) assay, ParA1 interacted with NtTTG1, and this interaction was robust in comparison with PCL1 and Lam, which were used as positive and negative controls, respectively (Fig. 2D). Second, oomycete elicitins all contain six Cys residues that form disulfide bonds and are indispensable for these proteins to function (Boissy et al., 1996; Bouaziz et al., 1994). Elicitins lose function if any single Cys is replaced with another amino acid, such as Ser (Kamoun et al., 1997). It has been predicted (Bouaziz et al., 1994; Jiang et al., 2006) that disulfide bonds could be formed between Cys residues located at sites 2 and 71 (2/71), 27/56 and 51/95 in the ParA1 sequence (Fig. 2C). In a model, Cys51 is positioned on the surface of the outer edge of the protein (Fig. 2C) and the position is important for protein-protein interaction (Boissy et al., 1996; Bouaziz et al., 1994; Walker et al., 1999). Consistently, the ParA1 mutant protein C51S (Fig. 1A), which failed to induce HR (Fig. 1B), also did not bind NtTTG1 in Y2H (Fig. 2D). Third, in NtTTG1 similarly as in AtTTG1, a Ser residue outside of the WD40 hydrophobic cavity (Fig. 2C) is thought to be crucial to the molecular interaction, because the substitution of Ser with phenylalanine nullifies the functions of AtTTG1 in *Arabidopsis* (Walker et al., 1999). Similar replacement of Ser94 in the NtTTG1 sequence resulted in the mutant protein S94F, which did not interact with ParA1 in Y2H (Fig. 2D). Lastly, proteins tested by Y2H had been fused to $His_{(6)}$ tag, a six tandem of histidine, and were subjected to an in vitro pulldown assay, which confirmed the interaction between ParA1 and NtTTG1 but not in other combinations (Fig. 2E). In this assay, the use of anti-His antibody detected any of the tested proteins or interactions between the proteins. ParA1 and NtTTG1 interaction was indicated by the two bands in the fifth loading panel in the gel, but no interactions were observed in other combinations (Fig. 2E). Clearly, ParA1 interacts in vitro with NtTTG1 and either Cys51 in the ParA1 sequence or Ser94 in the NtTTG1 sequence is crucial to the interaction.

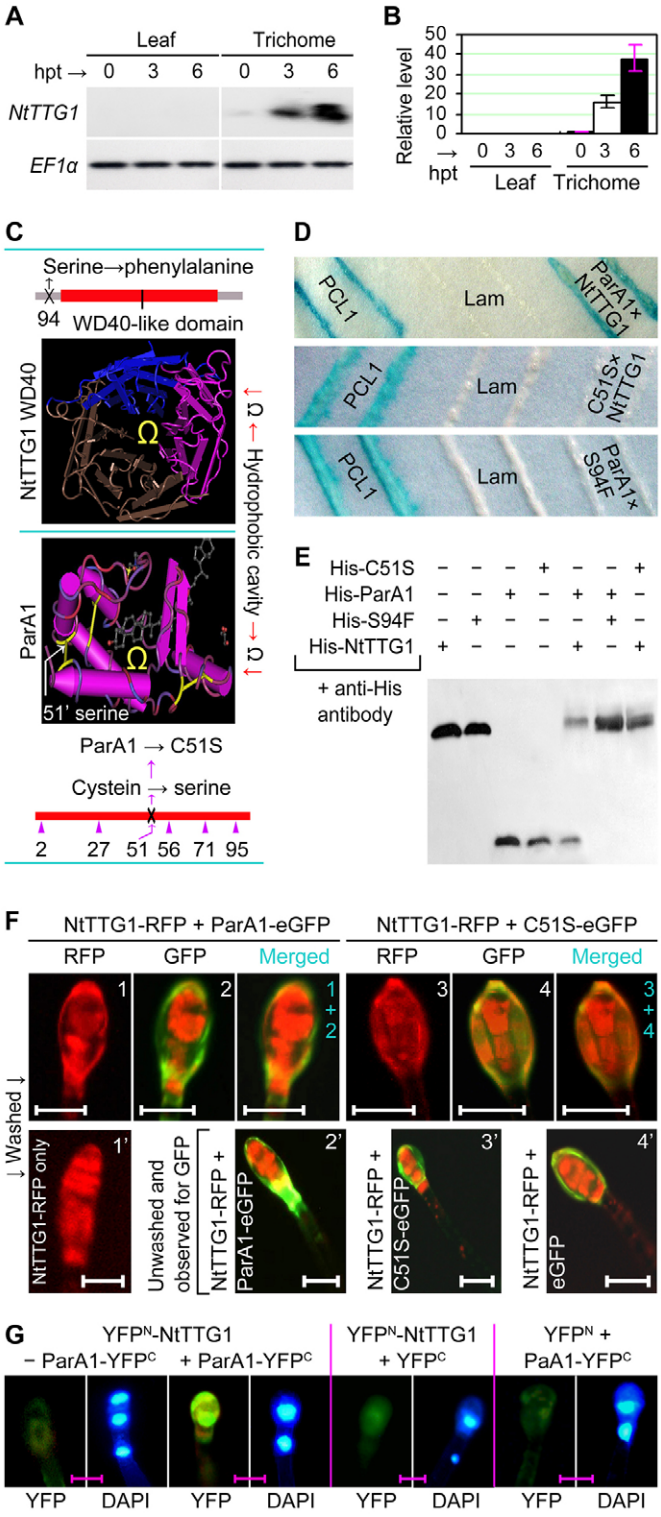

Fig. 2. Analyses of NtTTG1 and ParA1 interaction. (A,B) Northern blot analysis (using *EF1α* as a control) (A) and real-time RT-PCR measurement (mean ± s.d.) of *NtTTG1* expression at the indicated hpt with EVP (white histograms) or ParA1 (black histograms) (B). (C) Site-directed mutations (top and bottom linear diagrams) based on the structural modelling established with the programme at NCBI database. (D) Y2H assay for the protein combinations. PCL1, positive control; Lam, negative control. (E) An in vitro pulldown assay of ParA1 and NtTTG1. (F) Colocalization of transiently expressed NtTTG1 with ParA1, rather than C51S, at the trichome cell membrane. Trichomes on newly growing leaves at 5 days after plant transformation were treated (+) for 15 minutes with the fusion proteins. Treated trichomes were washed in water (1-4) or remained unwashed (1′-4′). RFP and GFP were visualized in contrast to dark and red backgrounds, respectively. (G) BiFC analysis of NtTTG1 and ParA1. In F and G, a photo represents 45 trichomes.

NtTTG1 interacts with ParA1 at leaf trichomes

We studied whether ParA1-NtTTG1 interaction could occur in leaf trichomes transiently expressing *NtTTG1* fused to a red-fluorescence protein (*RFP*) gene. Under direction of the cauliflower mosaic virus 35S promoter (35SP), *NtTTG1-RFP* was expressed strongly in trichomes and in mesophylls of transformed leaves within 12 hours after transformation (supplementary material Fig. S4). This constitutive *NtTTG1* expression conforms to the constitutive *AtTTG1* expression in *Arabidopsis* leaves (Zhao et al., 2008) but differs from ParA1-induced *NtTTG1* expression specifically in leaf trichomes of wild-type (WT) tobacco plants (Fig. 2A,B). Also unlike AtTTG1, which localizes preferentially to the nuclei of *Arabidopsis* trichomes (Zhao et al., 2008), NtTTG1-RFP was distributed over the cells of tobacco leaf trichomes (Fig. 2F, 1/1′). In 15 minutes after either ParA1-eGFP or C51S-eGFP were applied to leaf trichomes, both fusion proteins behaved like the control protein eGFP and both were distributed affluently in the trichomes around cell membranes (Fig. 2F, 2′-4′). However, in a protein-removing assay conducted on the basis of the water-soluble and diffusible property of elicitins (Huet et al., 1994; Huet et al., 1995; Kamoun et al., 1993; Yu, 1995), ParA1-eGFP remained tightly in trichomes but C51S-eGFP did not. When treated trichomes were washed with water, a marked proportion of C51S-eGFP was removed but ParA1-eGFP was not (Fig. 2F, 2/2′ and 4/3′; supplementary material Fig. S5), suggesting that ParA1-eGFP rather than C51S-eGFP might have diffused into the cells. Moreover, NtTTG1-RFP was apparently more concentrated around the cell membranes in the presence than the absence of ParA1-eGFP (Fig. 2F, 1/1′). When the photo images were analyzed for coincident NtTTG1 and ParA1 localization, NtTTG1-RFP was merged with ParA1-eGFP, instead of C51S-eGFP, at the membrane (Fig. 2F, 1+2/3+4). The in vivo interaction was revealed in a bimolecular fluorescence complementation (BiFC) assay (supplementary material Fig. S6). The assay detected a direct NtTTG1-ParA1 interaction around the cell membrane, instead of nuclei (Fig. 2G).

Leaf trichomes activate the HCD pathway before mesophylls

To correlate ParA1-NtTTG1 interaction with the priority of leaf trichomes to support HCD signal transduction over mesophylls, we characterized time courses of ROS burst, chromatin condensation and cell death induced in trichomes treated with ParA1, versus EVP, and in mesophylls underneath treated trichomes. ROS burst was evident in ParA1-treated trichomes at 1 hpt, 2 hours earlier than in mesophylls (Fig. 3A), spread from trichomes to mesophylls (Fig. 1C), and increased levels in both types of cells with time from 6 hpt to 12 hpt (supplementary material Fig. S7), at which time micro-HR appeared on leaves (Fig. 1D). As in mesophylls (Fig. 1C,D), the production of total ROS and H_2O_2 was also retarded in intact leaves (Fig. 3A).

Chromatin condensation, a hallmark of HCD (Alvarez et al., 1998; de Pinto et al., 2002), was studied by the 4,6-diamino-2-phenylindole (DAPI) fluoluminescence assay (Alvarez et al., 1998; de Pinto et al., 2002). Typical chromatin condensation (de Pinto et al., 2002) was found in trichomes and mesophylls following the

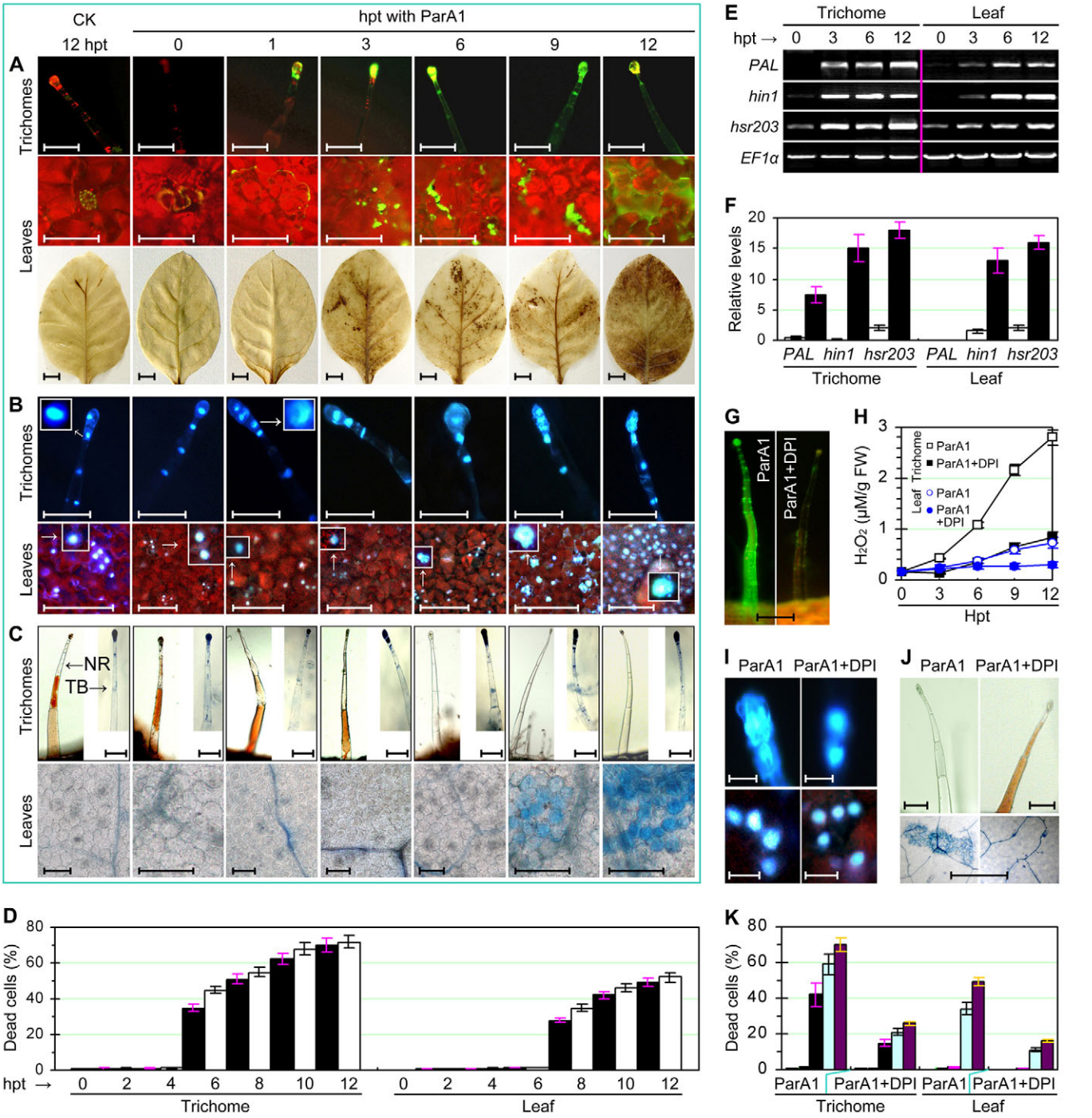

Fig. 3. HCD signal transduction from leaf trichomes to mesophylls. (A-C) Luminescence assays of H_2O_2 (A), chromatin condensation (B) and cell death (C) in treated trichomes, mesophylls underneath the trichomes and leaves. A photo represents 150 trichomes or 60 leaves. Dyes used in A were DCFH-DA (top two photo panels) and DAB (lower leaf panels). Insets in B are close-up views (3×) of the indicated nuclei. In C, trichomes were stained with neutral red (NR) or Trypan Blue (TB), and mesophylls were stained with TB. Scale bars: 3 cm (bottom row in A); 20 μm (top rows in A; B,C). (D) Percentage (mean ± s.d.) of cell death scored at a 1-hour interval subsequent to trichome treatment with ParA1. (E,F) RT-PCR analysis using *EF1α* as a control and real-time RT-PCR measurement (mean ± s.d.) of the three HCD marker genes expressed at the indicated hpt (E) and 6 hpt (F) with ParA1. (G-K) Comparisons of trichome treatments with ParA1 only and ParA1 applied together (+) with DPI in effects on the HCD signalling events and cell death. Plants were investigated at the indicated time points or 12 hpt. Scale bars: 20 μm. In H and K, error bars across tops of histograms represent s.d.

use of ParA1. Nuclei in cells of control (EVP-treated) trichomes and in cells of ParA1-treated trichomes within 1 hpt had a clear central nucleolus surrounded by a uniform stained chromatin, whereas, in cells of ParA1-treated trichomes over 1 hpt, chromatin had a swollen aspect and nuclei were cracked (Fig. 3B). Thus, chromatin condensation was induced in leaf trichomes by 1 hpt, ~5 hours earlier than in mesophylls, which did not show evident chromatin condensation until 6 hpt.

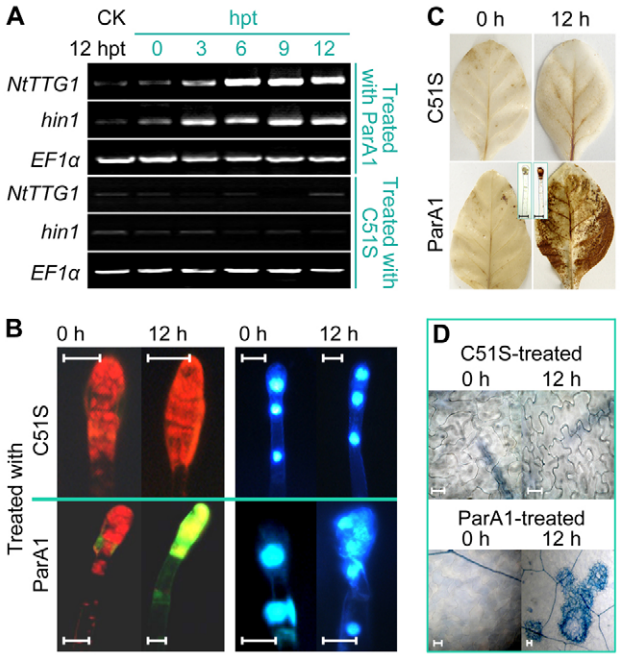

Fig. 4. The absence of HCD signalling events in trichomes and leaves of tobacco plants treated with C51S compared with ParA1. The absence of *NtTTG1* and *hin1* expression (A), H_2O_2 burst (B, left), chromatin condensation (B, right) and cell death (C,D) in leaf trichomes and the leaves following treatments of trichomes with C51S compared with ParA1. Scale bars: 20 μm.

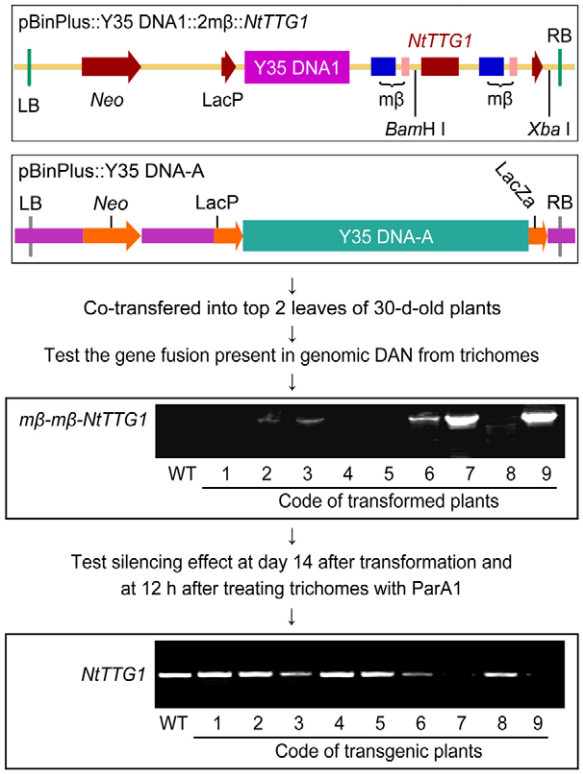

Fig. 5. Constructs and experimental procedure used to silence *NtTTG1*. Top two diagrams show the two-component gene-silencing constructs. The two gel panels represent one of 20 experimental repeats each containing nine transformed plants tested, compared with WT. Top gel panel is the result of PCR analysis of genomic DNA, and lower gel panel is result of RT-PCR analysis using RNA isolated from ParA1-treated trichomes. Codes of transformed plants are arbitrary. Plants coded as 7 and 9 as well as similar plants in another 19 repeat experiments were further investigated for the events shown in Fig. 6.

Cell death was surveyed. Initially, a Trypan Blue staining protocol widely used to visualize cell death in leaves (Alvarez et al., 1998; Peng et al., 2003) was used to visualize cell death in leaf trichomes. However, Trypan Blue stained the top first of five or six cells in the trichomes irrespective of treatment with EVP or ParA1 (Fig. 3C), causing uncertainty as to whether the staining sign indicated trichome cell death. We observed plasmolysis, an indicator of cell vitality, with the aid of neutral blue, which stains live cells (Weigel and Glazebrook, 2002). Both the absence of neutral red staining and the absence of glucose-induced plasmolysis of neutral-red-stained cells confirmed death of the cells vertically lower than the two top cells of trichomes (Fig. 3C). On the basis of observations using this method, death befell the lower 3-5 cells of ParA1-treated trichomes by 3 hpt, and the basal cells died by 6 hpt. Underneath, in mesophylls, cell death did not occur until 9 hpt, ~6 hours later than cell death in the trichomes (Fig. 3C). As well as the priority in time course, leaf trichomes also exceeded mesophylls in the levels of cell death (ANOVA tests, $P<0.01$) at each time point from 5 hpt (Fig. 3D). In leaf trichomes, HCD marker genes *PAL1*, *hin1* and *hsr203* (Chen et al., 2008; Peng et al., 2003; Peng et al., 2004) were expressed strongly from 3 hpt with ParA1 (Fig. 3E), whereas mesophylls were inferior to trichomes in time and extent of the gene expression (Fig. 3E,F). These results depict a course of time in HCD signal transduction from leaf trichomes to mesophylls.

This process accompanied the production of H_2O_2. In parallel experiments, the use of DPI to diminish the content of H_2O_2 (Riganti et al., 2004) decreased the amount of ROS in ParA1-treated trichomes and the underlying mesophylls (Fig. 3G). In both organs, H_2O_2 content declined significantly when ParA1 and DPI were applied together, compared with the use of only ParA1 (Student's *t*-test, $P<0.01$). When trichomes were treated with the ParA1+DPI combination versus only ParA1, H_2O_2 was 71% and 60% less in trichomes and leaves, respectively (Fig. 3H), and chromatin condensation was alleviated as well (Fig. 3I). Also, the use of DPI reduced the extent of cell death (Fig. 3J), not only retarding time of death but also decreasing the proportion of dead cells (Fig. 3K), in trichomes and subsequently in mesophylls. Thus, H_2O_2 is crucial for ParA1 to activate the HCD pathway in leaf trichomes.

C51S treatment and *NtTTG1* silencing inhibit HCD signalling in leaf trichomes

The HCD pathway was abrogated when leaf trichomes were treated with C51S. In contrast to marked expression of *NtTTG1* and *hin1* in leaf trichomes treated with ParA1, induced expression of both genes was eliminated in C51S-treated trichomes, compared with basal levels of the transcripts observed in controls (Fig. 4A). In C51S-treated trichomes, ROS burst and chromatin condensation did not occur; by contrast, nuclei appeared intact at 12 hpt, similarly as at 0 hpt, and ROS was not induced during that period (Fig. 4B). In mesophylls underneath C51S-treated trichomes, neither H_2O_2 nor cell death was evident relative to controls (Fig. 4C). Clearly, the HCD pathway was no longer activated when C51S was applied to trichomes.

The pathway was also nullified when *NtTTG1* was silenced via a virus-mediated RNA interference (RNAi) protocol (Tao and Zhou,

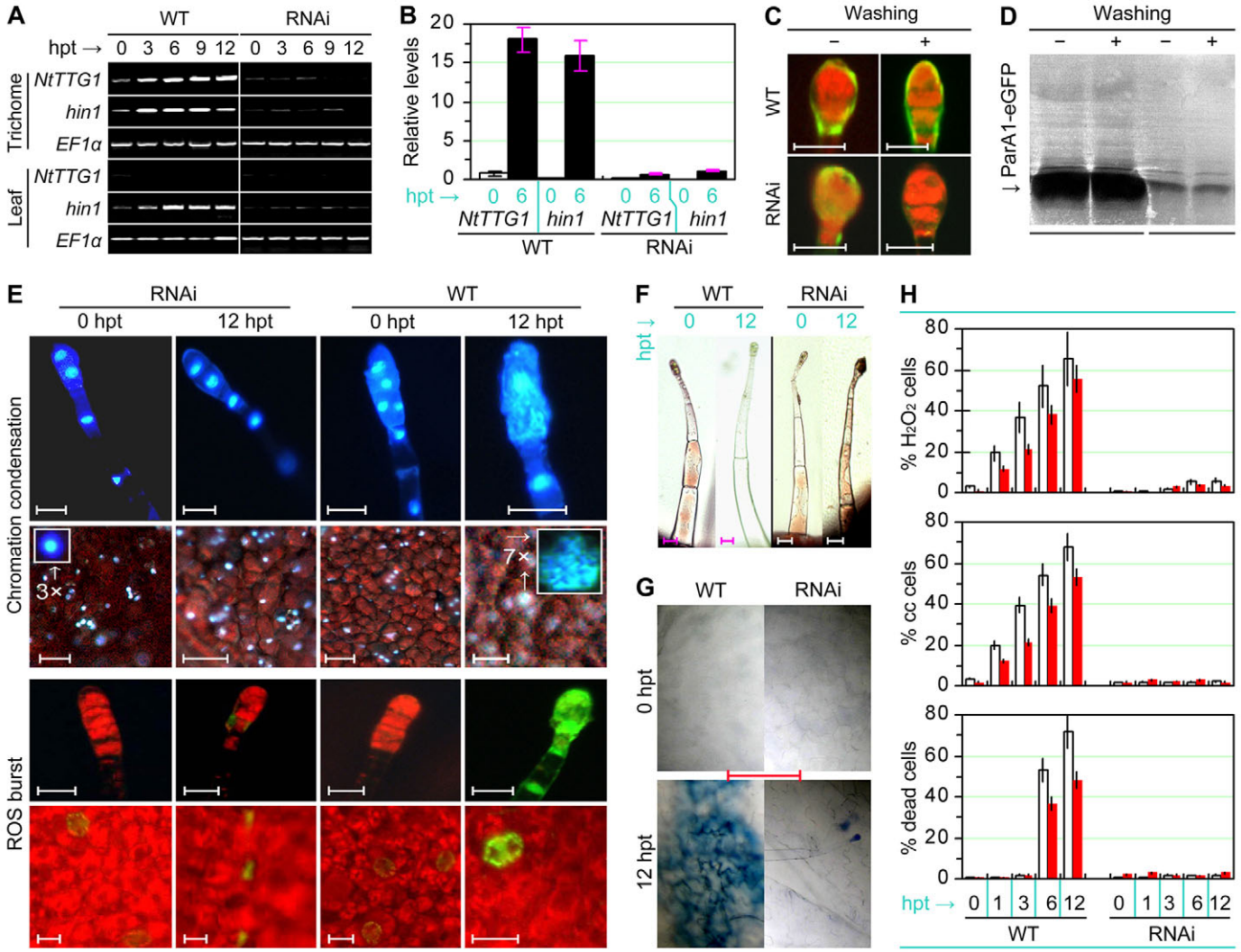

Fig. 6. The absence of NtTTG1-ParA1 interaction and ParA1-induced HCD signalling events in *NtTTG1*-silenced (RNAi) plants compared with WT. (A-G) ParA1-treated trichomes and/or the underlying mesophylls were investigated for gene expression (A), and quantified for means ± s.d. of relative levels (B), fluorescence of NtTTG1-ParA1 interaction (C), production of NtTTG1 (D), the HCD signalling events (E) in trichomes (top panels) and mesophylls (bottom panels), and cell death in trichomes (F) and leaves (G). In C, trichomes were treated for 15 minutes with ParA1-eGFP and then remained unwashed or were washed in water prior to fluoluminescence observation. In D, anti-His antibody was hybridized to a blot of proteins isolated from trichomes. Scale bars: 20 µm (C,E,F); 3 cm (G). (H) RNAi and WT trichome (white histograms) and mesophyll (red histograms) cell scoring in percentage (mean ± s.d.). cc, chromatin condensation.

2004). At 12 hours after plants were transformed with the silencing vector, the RNAi effect was observed as elimination or decrease in ParA1-induced expression of *NtTTG1* in leaf trichomes (Fig. 5). In these plants, RNAi cancelled the HR signalling events, which, however, were robust in WT plants tested in parallel experiments (Fig. 6). In RNAi plants, neither *NtTTG1* nor *hin1* was expressed following ParA1 application (Fig. 6A,B). In WT leaf-trichome cells, ParA1-eGFP that was applied externally was stably bound; however, the fusion protein was unable to remain in leaf trichomes of RNAi plants (Fig. 6C,D). RNAi plants did not support chromatin condensation (Fig. 6E), ROS (Fig. 6E) and H_2O_2 burst (supplementary material Fig. S8), and cell death (Fig. 6F,G), which did not occur over constitutive levels (Fig. 6G) in ParA1-treated trichomes and mesophylls underneath the trichomes. Therefore, NtTTG1 is crucial for ParA1 binding by leaf trichomes and for HR signal transduction from the trichomes to mesophylls.

Different elicitors induce chromatin condensation and cell death in leaf trichomes

To gain information about a broad importance of leaf trichomes in activating the HCD pathway, leaf trichomes were treated with different types of HCD elicitors versus specific control compounds, and treated trichomes and the underlying mesophylls were tested for chromatin condensation and cell death. As shown in Fig. 7, both events were observed similarly in WT and RNAi plants responding to the chemical elicitors, salicylic acid (Ryals et al., 1996) and paraquat (Ge et al., 2007), in contrast to water, or responding to proteinaceous harpin elicitors, $HrpN_{Ea}$ (Peng et al., 2003; Wei et al., 1992), $HpaG_{Xoo}$ (Liu et al., 2006) and $HpaG_{Xooc}$ (Chen et al., 2008), in contrast to EVP. Upon treatment with each elicitor, chromatin condensation and cell death appeared 4-6 hours earlier in leaf trichomes than in mesophylls. Riboflavin induces resistance to pathogens and does not induce HCD in tobacco (Dong and Beer,

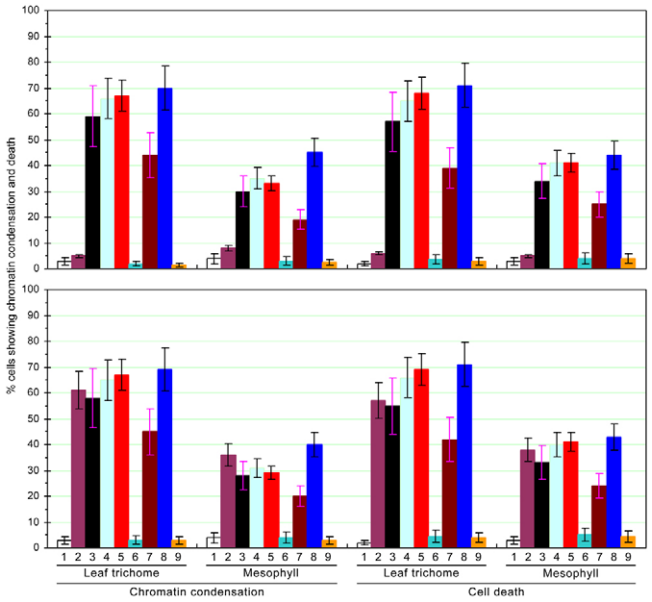

Fig. 7. Cell scoring (mean ± s.d.) at 12 hours after leaf trichomes of RNAi (top histograms) and WT (lower histograms) plants were treated with the indicated compounds. Compounds (codes on lower horizontal panel): 1, EVP; 2, ParA1; 3, $HrpN_{Ea}$; 4, $HpaG_{Xoo}$; 5, $HpaG_{Xooc}$; 6, water; 7, salicylic acid; 8, paraquat; 9, riboflavin.

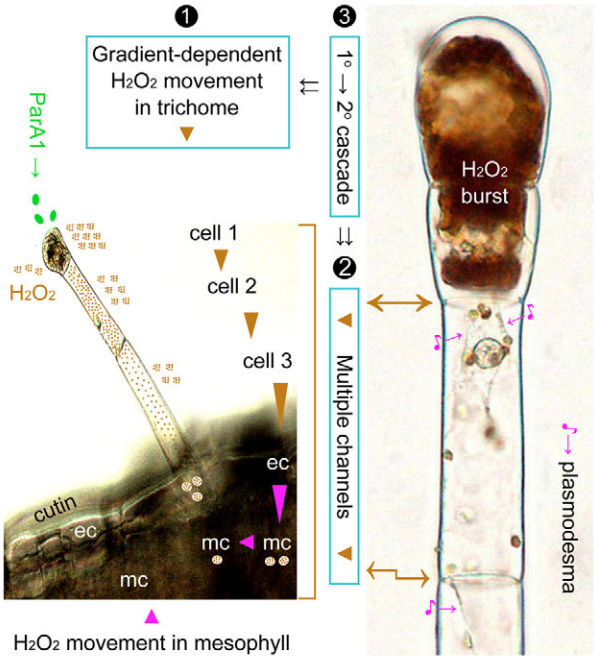

Fig. 8. A hypothesized model of the H_2O_2 signal transference in ParA1-treated trichome cells and from the trichome to mesophyll. Applying ParA1 to the surface of a trichome top cell induces H_2O_2 to accumulate in that cell. In conjecture, H_2O_2 is conveyed in sequence to the next trichome cell, epidermal cell (ec), and mesophyll cell (mc) underneath the trichome. This process may comply with three mechanisms numbered as function in order. The '1°→2° cascade', assumed to promote cell-to-cell H_2O_2 shift via plasmodesmata or other channels, refers to the possibility that HCD induced in a cell causes adjacent cells to incur HCD and such an HCD cascade acts to elicit additional H_2O_2 generation in adjacent cells that are still alive. Left image is from a leaf without any treatment; presumable H_2O_2 is depicted as superimposed brown dots, and the number of dot mass indicates the speculated gradient of H_2O_2 concentration. The partial trichome shown on the right has H_2O_2 accumulation visualized as brown precipitate by DAP staining at 1 hpt with ParA1 applied to the top cell. Quavers indicate locations of plasmodesma.

2000). Consistently, neither chromatin condensation nor cell death was evident in riboflavin-treated trichomes and mesophylls underneath the trichomes (Fig. 7). By contrast, significant levels of chromatin condensation and cell death were induced by other elicitors as were induced by ParA1 in comparison with water or EVP (ANOVA tests, $P<0.01$). In response to the elicitors, but not to ParA1, levels of condensation and cell death were similar in leaf trichomes or mesophylls of RNAi and WT plants (Fig. 7).

Discussion

Trichomes on the surfaces of aerial parts of plants are distributed like biological antenna exposed to a variety of environmental cues (Roy et al., 1999; Wagner, 1991) and serve as the first procession for plants to encounter multiple extrinsic challenges (Amme et al., 2005; Loughner et al., 2008). Growing at the most external parts of plants, leaf trichomes are assumed to function as a channel to connect the extrinsic signals with intrinsic plant signalling pathways (Amme et al., 2005; Kanzaki et al., 2008; Roy et al., 1999; Traw and Bergelson, 2003). This notion has been tested in the present study by investigating the several events that are crucial to HCD signal transduction in tobacco leaf trichomes and mesophylls following localized treatment of trichomes with ParA1, a particular elicitin protein (Kamoun et al., 1993).

ParA1 produced by *P. parasitica* is one of more than 30 elicitins identified so far from more than 20 species of plant pathogenic oomycetes (Liang et al., 2004; Yu, 1995). Some elicitins, such as INF1 of *Phytophthora infestans* (Kanzaki et al., 2008), act as avirulence factors in host plants and as inducers of HCD and other defensive mechanisms, such as systemic acquired resistance (SAR), in non-host plants of the elicitin-producing oomycetes (Bonnet et al., 1996; Colas et al., 2001; Kamoun et al., 1993; Kamoun, 2001; Liang et al., 2007). ParA1 can be produced by the variety *nicotianae* and other varieties of *P. parasitica* (Kamoun et al., 1993; Ricci et al., 1992). There is no evidence to conclude any role of ParA1 in pathogenicity; but instead, the protein is specified as an elicitor of HCD and SAR in plants that are hosts of *P. parasitica* (Kamoun et al., 1993; Liang et al., 2007). Results obtained from this study confirm our previous observation (Liang et al., 2007) that the application of *P. parasitica* var. *nicotianae* ParA1 induces the production of ROS, in particular of H_2O_2, and HCD in tobacco. The failure of C51S to fulfil the function conforms to the importance of Cys residues in elicitin sequences. Disulfide bonds formed by these residues are indispensable for elicitins to function in plants (Boissy et al., 1996; Bouaziz et al., 1994). Locations of Cys residues and predicted disulfide bonds are the same in ParA1 produced by different varieties of *P. parasitica* (Kamoun et al., 1993). These elicitins lose functions even if a single Cys is replaced with Ser at any location, such as Cys51 manipulated in this study and Cys27 tested previously (Kamoun et al., 1997). Therefore, ParA1 is desired for use to study molecular mechanisms that underlie plant responses to the external application of elicitins.

In response to ParA1, leaf trichomes and mesophylls sequentially increase levels of ROS, and especially H_2O_2, a cellular signal that regulates the HCD pathway (Alvarez et al., 1998; Torres et al., 2006). The elevation of H_2O_2 and subsequent cell death in leaf

trichomes and mesophylls suggest a connection between both types of cells in the signal transduction. Concomitantly, the use of ParA1, rather than C51S, induces the expression of *NtTTG1*, a homologue of the *Arabidopsis AtTTG1* gene, which is crucial to trichome morphogenesis (Morohashi et al., 2007; Walker et al., 1999; Zhao et al., 2008). ParA1-induced *NtTTG1* expression is restricted to leaf trichomes. This suggests that *NtTTG1* is involved in leaf-trichome response to ParA1 and that *NtTTG1* differs from *AtTTG1*, which is expressed in both trichomes and mesophylls (Zhao et al., 2008). The AtTTG1 protein also differs from NtTTG1 in its subcellular localization. AtTTG1 localizes preferentially to nuclei of trichomes but NtTTG1 is distributed throughout trichome cells in the absence of ParA1 treatment and becomes affluent at the membranes of leaf-trichome cells following the application of ParA1. The morphology and density of leaf trichomes are similar between WT and RNAi plants (data not shown), so the role of NtTTG1 in trichome development remains characterized. However, several assays suggest that NtTTG1 is a ParA1-interacting protein and that both proteins interact in leaf trichomes. Analysis of RNAi plants demonstrates that NtTTG1 and its interaction with ParA1 are required for the activation of the HCD pathway in leaf trichomes.

In response to an elicitor, the HCD pathway is activated concomitantly with the elevation in cellular H_2O_2 level and chromatin condensation is regarded as a hallmark of the pathway (Alvarez et al., 1998; Peng et al., 2003; Torres et al., 2006). Nevertheless, causal relationships between these events might vary with circumstances, such as types of elicitors (Sasabe et al., 2000; Van Breusegem and Dat, 2006; Yu, 1995). The time course for ParA1 to induce ROS (particularly H_2O_2) production, chromatin condensation and cell death suggests that leaf trichomes come before mesophylls in activating the HCD pathway. The compromise of the pathway by DPI and catalase indicates the requirement for H_2O_2 in trichome response to ParA1. The pharmacological study was carried out according to the reviewers who scrutinized this article with an advice that experimental evidence minimized the role of ROS in elicitin-induced cell death (Dorey et al., 1999; Binet et al., 2001; Rustérucci et al., 1999). To our knowledge, causal relationships between ROS and cell death and between cell death and plant defence against pathogens remain an issue with broad importance. In both plants under infection by pathogens (Jabs et al., 1996; Tanaka et al., 2003) or those treated with different elicitors (Desikan et al., 1998; Peng et al., 2003; Peng et al., 2004; Tanaka et al., 2003; Trouvelot et al., 2008), ROS production is either dispensable for the induction of cell death and indispensable for the defence response. This is true for elicitins based on published studies.

We browsed more than 120 papers relating to elicitins published April 1981 to March 2009. Many of the papers touched on causal relationships between ROS and HCD or the defence response. They were concomitant, but the consequence was unclear in most reports (Bourque et al., 1998; Fleischmann et al., 2005; Kanzaki et al., 2008; Koehl et al., 2007; Lebrun-Garcia et al., 1998; Lecourieux et al., 2002; Liang et al., 2007; Lochman et al., 2005; Rusterucci et al., 1996; Yoshioka et al., 2003). Several reports argued that ROS or H_2O_2 was elevated but was not required for the elicitation of HCD and defence response in tobacco following treatment with *Phytophthora megasperma megaspermin* (Dorey et al., 1999) or *P. cryptogea cryptogein* (Binet et al., 2001; Rustérucci et al., 1999). However, *cryptogein*-induced ROS production was correlated with subsequent cell death in tobacco epidermis (Allan and Fluhr, 1997). Evidence also showed interactive signalling components in the regulation of tobacco cell death and defence response induced by INF1 (Kanneganti et al., 2006; Kamoun et al., 1998; Sasabe et al., 2000; Takemoto et al., 2005). INF1 induces H_2O_2 generation via an NADPH oxidase and this contributes to plant resistance against pathogens (Asai et al., 2008). The development of plant resistance requires protein kinases, which are also involved in the induction of ROS and HCD (Asai et al., 2008; Sasabe et al., 2000). These studies accepted or denied the role of ROS in HCD mainly on the basis of chemical and/or genetic analyses of plant leaves or cultured plant cells, instead of trichomes.

Evidence from both pharmacological and genetic analyses in our study supports the important role of H_2O_2 in ParA1-induced activation of the HCD pathway in tobacco leaf trichomes. Both types of analyses show a course of time for ROS, and especially H_2O_2, to accumulate in ParA1-treated trichomes and then spread from the trichomes to the epidermal and mesophyll cells, suggesting a connection of HCD signalling between trichomes and mesophylls. H_2O_2 occupies a prominent proportion of the produced ROS induced by ParA1, as observed in this study, and induced by other elicitins investigated previously (Allan and Fluhr, 1997; Ashtamker et al., 2007). In response to INF1, for example, ROS are generated within leaf epidermal cells and quickly moves as H_2O_2 into neighbouring cells (Allan and Fluhr, 1997), consistent with the ROS behaviour in response to ParA1.

Although we have no direct evidence to state how H_2O_2 moves in trichome cells and how it is conveyed to mesophylls, our luminescence assays combined with genetic and pharmacological analyses, considered together with previous studies, suggest that H_2O_2 transference might comply with three mechanisms (Fig. 8). Presumably, concentration gradient is sufficient for the small and diffusible H_2O_2 molecule to shuttle within a cell and across cells. When cultured tobacco cells are treated with cryptogein, the generation of H_2O_2 is affluent first in the nuclear region and, after a short delay, at the cell periphery (Ashtamker et al., 2007), suggesting a concentration-dependent diffusion. This is supposed as the first wave of H_2O_2 movement. Second, cell-to-cell H_2O_2 movement is likely to occur via multiple cellular devices. Besides plasmodesmata and apoplastic sites observed early in the epidermis of cryptogein-treated tobacco plants (Allan and Fluhr, 1997), several other cellular devices scrutinized recently might also allow for cell-to-cell transportation of different types of ROS, such as $O_2^{\bullet -}$ and H_2O_2. Both types of ROS can move across the plasma membranes via distinct channels, whereas H_2O_2 prefers aquaporin channels for diffusion to elicit intracellular signalling response (Fisher, 2009), indicating the possibility of the signal shift across water-permeable plant cell walls. The third mechanism that promotes H_2O_2 transference possibly comes from a cascade of cellular response to ParA1 treatment, i.e. HCD first induced in a cell causes adjacent cells to incur HCD and such a cascade acts to elicit additional H_2O_2 generation in adjacent cells that are still alive. This hypothesized consequence must facilitate the speculated process of cell-to-cell trafficking of the H_2O_2 signal. Further studies to inspect these possibilities will increase our understanding of signalling regulation for plant responses to elicitins in general and for the ParA1-activated HCD pathway in particular.

The abrogation of the pathway in *NtTTG1*-silenced plants and in C51S-treated plants as well as the absence of ParA1-TTG1 interaction in both cases suggests that the interaction is crucial to HCD signal transduction in leaf trichomes. Similar signalling processes can be induced by different types of HCD elicitors, including harpin proteins of plant pathogenic bacteria (Chen et al.,

2008; Dong et al., 1999; Liu et al., 2006; Wei et al., 1992) and the chemical elicitors SA (Ryals et al., 1996) and paraquat (Ge et al., 2007). The proteinaceous and chemical elicitors induce chromatin condensation and cell death first in leaf trichomes and then in mesophylls in RNAi plants, as they do in WT. This result suggests that leaf trichomes play a broad role in communicating the extrinsic signals to the intrinsic HCD signalling pathway. This result also suggests that the function of NtTTG1 in the HCD pathway is specific for ParA1.

The significance of the NtTTG1-ParA1 interaction could be considered in three perspectives. First, it represents a mode of leaf-trichome sensing of the stimulation by elicitins applied extrinsically but has not been correlated to plant sensing of elicitins released by pathogenic oomycetes during infection. Thus, the significance of NtTTG1-ParA1 interaction differs from the elicitin–lipid-transfer-protein–sterol triple complex, which functions during oomycete infection (Blein et al., 2002; Boissy et al., 1999; Osman et al., 2001). Oomycetes require sterols for growth but cannot synthesize these compounds by themselves (Ponchet et al., 1999). During infection of plants, sterols required for pathogenic oomycetes to growth are supplied via the triple complex, which captures and carries sterols diffused from the host plants and then transports the compounds to oomycete cells (Blein et al., 2002; Mikes et al., 1998; Vauthrin et al., 1999). Second, regarding molecular recognition and subsequent response, the NtTTG1-ParA1 interaction differs from plant perception to some other elicitins, such as INF1 (Kanzaki et al., 2008) and cryptogein (Lochman et al., 2005; Pugin et al., 1997); both elicitins have been shown to induce H_2O_2 production and HCD by activating plant signalling processes independent of sterol binding. In tobacco, for example, a lectin-like receptor-kinase protein functions to bind INF1 and the binding activity is required for INF1 to induce H_2O_2 and HCD (Zhao et al., 2008). Hence, different plant proteins are required for interactions with different types of elicitins during the distinct processes, such as oomycete infection and plant response to the extrinsic application of elicitins (Blein et al., 2002; Kanzaki et al., 2008; Lochman et al., 2005). Finally, considering the diversity of plant extrinsic cues, one could believe that NtTTG1-ParA1 interaction presents an example that trichomes function like biological antennae to sense outside signals. Trichomes always encounter numerous diverse challenges from the environment (Traw and Feeny, 2008), whereas NtTTG1 is specific in sensing ParA1, at least among the proteinaceous elicitors we have tested. Therefore, many other regulators of trichome development (Morohashi et al., 2007; Zhao et al., 2008), besides TTG1, should be rational to convey outside signals inwards to particular intracellular pathways. We feel that this notion is worthy of further study.

Materials and Methods

ParA1 manipulation

The *parA1* gene was cloned by PCR with genomic DNA of *P. parasitica* var. *nicotianae*. Primers were 5′-CGG**CATATG**AACTTCCGCGCTCTGTTCGC-3′ and 5′-CGGCCATGGCAGTGACGCGCACGTAGACG-3′ synthesized according to the reported sequence (Kamoun et al., 1993) of *parA1* (GenBank accession number S67432); bold and underlined letters are *Nde*I and *Nco*I restriction sites, respectively. Confirmed gene clones were subjected to prokaryotic transformation and expression (Chen et al., 2008; Liang et al., 2007). The mutant *C51S* was obtained through site-directed mutagenesis (Liu et al., 2006) conducted by PCR with the MutanBEST Kit (Takara Biotech, Dalian, China) and specific primers 5′-TCCGCGTCGACGGCGTCCAA-3′ and 5′-CATGAGCTTGTACTGCTCCGTCGTGGG-3′. The *GFP* gene used to construct *C51S-eGFP* was cloned from the vector pEGFP-C1 (Clontech Laboratories, Palo Alto, CA). Primers were 5′-CGG**CCATGG**ATGGTGAGCAAGGGCGAG-3′ and 5′-CGGGTCGACCTTGTACAGCTCGTCCATGC-3′; bold and underlined letters are *Nco*I and *Sal*I restriction sites, respectively. Each correct sequence of *parA1* and the derivates was inserted into the pET30a(+) vector (EMD Bioscience, Darmstadt, Germany) between *Nde*I and *Nco*I sites to reserve the upstream histidine tag (His-tag) close to the *T7* promoter; the downstream His-tag was excluded by adding proper stop codons in the primers. After transformation and sequencing, recombinant *E. coli* BL21 cells were incubated to produce protein preparations (Chen et al., 2008). Proteins were quantified (Liu et al., 2006) and used at 10 μg/ml.

Trichome treatment

Plants were grown as described (Peng et al., 2004) for 35 days before use in all the experiments. Localized treatment of uniform leaf trichomes on plants was performed with the aid of Inject Man NI2 Micromanipulator and FemtoJet Microinjector Systems (Eppendorf, Hamburg, Germany). A glass syringe connected to the microinjector device was loaded with 5 μl of an aqueous solution of proteins, chemicals or their combinations to be tested. The solution was applied to the surface of the top first cell of a leaf trichome under monitoring with the micromanipulator.

Manipulation of *NtTTG1*

The gene was cloned by reverse transcriptase (RT)-PCR using RNA isolated from ParA1-treated tobacco and specific primers designed according to the sequence of *AtTTG1* (GenBank accession number NM_180739). Primers were 5′-ATGGATAATTCAGCTCCAGATTC-3′ and 5′-TCAAACTCTAAGGAGCTGCAT-3′. *S94F* was obtained through site-directed mutagenesis (Liu et al., 2006) conducted by PCR with the MutanBEST Kit (Takara Biotechnology, Dalian, China) and specific primers 5′-CGTCCTTCCTTCGGAGATCTC-3′ and 5′-ACGGAGAGAAGGACTGAAC-3′. The *GFP* gene used to construct *ParA1-eGFP* was cloned as described above. The *RFP* gene used to construct *NtTTG1-RFP* was cloned from the vector pDsRed2-1 (Clontech); primers were 5′-CGG*G***AATTC**ATGGCCTCCTCCGAGAACGTC-3′, 5′-CGGGTCGACCTACAGGAACAGGTGGTGGCGG-3′; bold and underlined letters are *Eco*RI and *Sal*I restriction sites, respectively. Regular protocols (Chen et al., 2008; Peng et al., 2004) were used in gene recombination.

Gene-silencing experiment

Tobacco curly shoot virus-mediated RNAi was performed using vectors pBinPlus-Y35 DNA1::2mβ and pBinPlus::Y35 DNA-A (Tao and Zhou, 2004). A 500-bp fragment of *NtTTG1* was cloned by PCR using tobacco genomic DNA and specific primers 5′-CG**CCCGGG**GCCGTTGACTTCCTTCGATTG-3′ and 5′-CGTCTAGAGGTCCAGCAACAGTAGGAAGC-3′; bold and underlined letters refer to *Sma*I and *Xba*I restriction sites, respectively. Confirmed product was ligated into pBinPlus-Y35 DNA1::2mβ by restrictions and ligation to create pBinPlus-Y35 DNA1::2mβ::*NtTTG1*. The unit was transferred into *Agrobacterium tumefaciens* EHA105 cells (Peng et al., 2004). A suspension of *NtTTG1*-containing recombinant EHA105 cells in mixture with equal volume of EHA105 cells already transformed with pBinPlus::Y35 DNA-A was infiltrated into emerging heart leaves of 30-day-old tobacco plants (Tao and Zhou, 2004). Control plants were infiltrated with pure water.

Y2H and pulldown assays

The Clontech Y2H system was used and all the operations followed instructions by the vender (Clontech Laboratories, Palo Alto, CA). *parA1* or *C51S* used as bait was cloned in the plasmid pGBKT7, and *NtTTG1* or *S94F* was cloned into pGADT7 to make preys. Combinations of pGBKT7::parA1 × pGADT7::NtTTG1, pGBKT7::C51S × pGADT7::NtTTG1 and pGBKT7::parA1 × pGADT7::S94F were transferred separately into cells of *Saccharomyces cerevisiae* strain Y190. The provided PCL1 and pGADT7-T/Lam were used as positive and negative controls, respectively. Clones growing on the amino-acid-deficient medium SD-4 were subjected to the β-galactosidase assay. In vitro pulldown assay was conducted with the ProFound GST Pull-down Assay System as per protocol provided by the manufacturer (PIERCE, Rockford, IL). *NtTTG1* and *S94F* were cloned separately into the pET41a(+) vector that carries a $His_{(6)}$ tag, and *parA1* and *C51S* were cloned separately into the pET30a(+)::$His_{(6)}$ vector. His-fused proteins were produced by prokaryotic expression (Chen et al., 2008). The four fusion proteins were assembled as three pair combinations (GST-NtTTG1/His-ParA1, GST-NtTTG1/His-C51S and GST-S94F/His-ParA1), loaded into the Handee Spin Column, and eluted with the glutathione elution buffer. Eluted proteins were subjected to electrophoresis in sodium dodecyl sulphate-polyacrylamide gel amended with 30% tricine. Gel blots were hybridized with monoclonal His antibody (Merck Chemicals, Darmstadt, Germany).

BiFC experiment

The *YFP* gene used in BiFC was cloned from the plasmid pCAG-YFP (Addgene, Cambridge, MA). Primers specific for YFP^N were 5′-**TCTAGA**cgctccatcgccacgATGGTGAGCAAGGGCGAGGAGCTGTTCACCG-3′ and 5′-CTGCAGTTAGGCCATGATATAGACGTTGTGGCTG-3′. Primers used to clone YFP^C were 5′-**GAATTC**cgcccggcctgcaagatcccgaacgacctgaaacagaaggtcatgaaccacGACAAGCAGAAGAACGGCATCAAGGTG-3′ and 5′-AAGCTTTTACTTGTACAGCTCGTCCATGCCGAG. Primers specific for *NtTTG1* and used to construct *NtTTG1-YFP*^{N} were 5′-**GGTACC**ATGGATAATTCAGCTCCAGATTCG-3′ and 5′-GGATCCAACTCTAAGGAGCTGCATTTTGTTAG-3′. Primers specific for *ParA1* and used to

construct *ParA1-YFP*^{C} were 5′-**GGTACC**ATGAACTTCCGCGCTCTGTTCGC-3′ and 5′-GGATCCCAGTGACGCGCACGTAGACGAGAAC-3′. In sequences of these primers, bold and underlined letters are *Kpn*I and *Bam*HI restriction sites, respectively; letters in lower case are coding sequences for the peptides used as linkers in the construction (supplementary material Fig. S5). Construction of NtTTG1-YFP^{N} and ParA1-YFP^{C} followed a standard method (Bracha-Drori et al., 2004; Feng et al., 2008). *NtTTG1-YFP*^{N} was transferred into the plant transformation and expression vector pCAMBIA1301, and the resulting unit was transferred into *A. tumefaciens* EHA105 cells, followed by transformation of top second and third leaves of tobacco; regular methods (Peng et al., 2004) were used during these operations. *ParA1-YFP*^{C} was subjected to prokaryotic expression (Chen et al., 2008). ParA1-YFPC preparation was applied to the top first cells of trichomes on transformed leaves 2 days after transformation. Only YFP^{N} and YFP^{C} manipulated as NtTTG1-YFP^{N} and ParA1-YFP^{C} were used as controls. Protein-treated trichomes 15 minutes after treatment were washed in water and observed by microscopy.

Luminescence imaging

Images in luminescence assays were focused on leaves, mesophyll tissues or trichomes photographed using digital cameras Leica DC300F and Olympus C-7070 Wide Zoom connected with fluorescence microscope Leica DMR and fluorescence binocular microscope Olympus SZX 12, respectively. Microscopes were driven by visible light to observe chemiluminescence of leaves stained with DAB, neutral red or Trypan Blue. Fluoluminescence was observed using a hydrargyrum light (Hg 50 W). Wave lengths of excitation filters and emission light were 330-385 and 456 nm for DAPI, and 460-490 and 525 nm for DCFH-DA. Fluoluminescence of both dyes was visualized in contrast to red background. An excitation filter for 460-490 nm and emission light of 509 nm were used to capture GFP fluoluminescence under red background. Imaging of RFP was performed with excitation at 545-580 nm and emission at 583 nm in contrast to dark background. An excitation filter for 460-490 nm and emission light of 527 nm were used to capture YFP fluoluminescence under red background.

Others

Experimental procedures for protein production and bioassay, leaf staining, gene expression analysis, measurement of H_2O_2 content, assays of leaf trichome and mesophyll responses to multiple elicitors, experimental scale and data analysis can be obtained for the authors on request.

We thank Xueping Zhou for the gift of gene-silencing vectors and the two anonymous reviewers for scrutinizing the manuscript. This study was supported by National Science Foundation for Distinguished Young Scholars (grant no. 30525088), National Development Plan of Key Basic Scientific Studies (973 Plan) Project 2 (2006CB101902), Natural Science Foundation (30771441) and National High-Technology Development (863) Plan (2006AA10Z430) in China.

References

Achard, P., Vriezen, W. H., Van Der Straeten, D. and Harberd, N. P. (2003). Ethylene regulates Arabidopsis development via the modulation of DELLA protein growth repressor function. *Plant Cell* **15**, 2816-2825.

Ahn, I. P., Kim, S., Lee, Y. H., Suh, S. C. (2007). Vitamin B1-induced priming is dependent on hydrogen peroxide and the *NPR1* gene in Arabidopsis. *Plant Physiol.* **143**, 838-848.

Allan, A. C. and Fluhr, R. (1997). Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* **9**, 1559-1572.

Alvarez, M. E., Pennell, R. L., Meijer, R. J., Ishikawa, A., Dixon, R. A. and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* **92**, 773-784.

Ambrósio, S. R., Oki, Y., Heleno, V. C., Chaves, J. S., Nascimento, P. G., Lichston, J. E., Constantino, M. G., Varanda, E. M. and Da Costa, F. B. (2008). Constituents of glandular trichomes of *Tithonia diversifolia*: relationships to herbivory and antifeedant activity. *Phytochemistry* **69**, 2052-2060.

Amme, S., Rutten, T., Melzer, M., Sonsmann, G., Vissers, J. P., Schlesier, B. and Mock, H. P. (2005). A proteome approach defines protective functions of tobacco leaf trichomes. *Proteomics* **5**, 2508-2518.

Asai, S., Ohta, K. and Yoshioka, H. (2008). MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in *Nicotiana benthamiana*. *Plant Cell* **20**, 1390-1406.

Ashtamker, C., Kiss, V., Sagi, M., Davydov, O. and Fluhr, R. (2007). Diverse subcellular locations of cryptogein-induced reactive oxygen species production in tobacco Bright Yellow-2 cells. *Plant Physiol.* **143,** 1817-1826.

Baillieul, F., de Ruffray, P. and Kauffmann, S. (2003). Molecular cloning and biological activity of α-, β-, and γ-megaspermin, three elicitins secreted by *Phytophthora megasperma* H20. *Plant Physiol.* **131**, 155-166.

Balestrasse, K. B., Zilli, C. G. and Tomaro, M. L. (2008). Signal transduction pathways and haem oxygenase induction in soybean leaves subjected to salt stress. *Redox Rep.* **13**, 255-262.

Binet, M. N., Humbert, C., Lecourieux, D., Vantard, M. and Pugin, A. (2001). Disruption of microtubular cytoskeleton induced by cryptogein, an elicitor of hypersensitive response in tobacco cells. *Plant Physiol.* **125**, 564-572.

Blein, J. P., Coutos-Thévenot, P., Marion, D. and Ponchet, M. (2002). From elicitins to lipid-transfer proteins: a new insight in cell signalling involved in plant defence mechanisms. *Trends Plant Sci.* **7**, 293-296.

Blokhina, O., Virolainen, E. and Fagerstedt, K. V. (2003). Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot. Lond.* **91**, 179-194.

Boissy, G., de La, Fortelle, E., Kahn, R., Huet, J. C., Bricogne, G., Pernollet, J. C. and Brunie, S. (1996). Crystal structure of a fungal elicitor secreted by *Phytophthora cryptogea*, a member of a novel class of plant necrotic proteins. *Structure* **4**, 1429-1439.

Boissy, G., O'Donohue, M., Gaudemer, O., Perez, V., Pernollet, J. C. and Brunie, S. (1999). The 2.1 A structure of an elicitin-ergosterol complex: a recent addition to the Sterol Carrier Protein family. *Protein Sci.* **8**, 1191-1199.

Bonnet, P., Bourdon, E., Ponchet, M., Blein, J. P. and Ricci, P. (1996). Acquired resistance triggered by elicitins in tobacco and other plants. *Eur. J. Plant Pathol.* **102**, 181-192.

Bouaziz, S., Van Heijenoort, C., Guittet, E., Huet, J. C. and Pernollet, J. C. (1994). Resonance assignment, cysteine-pairing elucidation and secondary-structure determination of capsicein, an α-elicitin, by three-dimensional ^{1}H-NMR. *Eur. J. Biochem.* **220**, 427-438.

Boughton, A. J., Hoover, K. and Felton, G. W. (2005). Methyl jasmonate application induces increased densities of glandular trichomes on tomato, *Lycopersicon esculentum*. *J. Chem. Ecol.* **31**, 2211-2216.

Bourque, S., Ponchet, M., Binet, M. N., Ricci, P., Pugin, A. and Lebrun-Garcia, A. (1998). Comparison of binding properties and early biological effects of elicitins in tobacco cells. *Plant Physiol.* **118**, 1317-1326.

Bouyer, D., Geier, F., Kragler, F., Schnittger, A., Pesch, M., Wester, K., Balkunde, R., Timmer, J., Fleck, C. and Hulskamp, M. (2008). Two-dimensional patterning by a trapping/depletion mechanism: the role of TTG1 and GL3 in *Arabidopsis* trichome formation. *PLoS Biol.* **6**, 1166-1177.

Bracha-Drori, K., Shichrur, K., Katz, A., Oliva, M., Angelovici, R., Yalovsky, S. and Ohad, N. (2004). Detection of protein-protein interactions in plants using bimolecular fluorescence complementation. *Plant J.* **40**, 419-427.

Calo, L., Garcia, I., Gotor, C. and Romero, L. C. (2006). Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a *Trichoderma* α-1,3-glucanase. *J. Exp. Bot.* **57**, 3911-3920.

Chen, L., Qian, J., Qu, S. P., Long, J. Y., Yin, Q., Zhang, C. L., Wu, X. J., Sun, F., Wu, T. Q., Hayes, M. et al. (2008). Identification of specific fragments of $HpaG_{Xooc}$, a harpin from *Xanthomonas oryzae* pv. *oryzicola*, that induce disease resistance and enhance growth in plants. *Phytopathology* **98**, 781-791.

Colas, V., Conrod, S., Venard, P., Keller, H., Ricci, P. and Panabieres, F. (2001). Elicitin genes expressed *in vitro* by certain tobacco isolates of *Phytophthora parasitica* are down regulated during compatible interactions. *Mol. Plant Microbe Interact.* **14**, 326-335.

Costet, L., Dorey, S., Fritig, B. and Kauffmann, S. (2002). A pharmacological approach to test the diffusible signal activity of reactive oxygen intermediates in elicitor-treated tobacco leaves. *Plant Cell Physiol.* **43**, 91-98.

de Pinto, M. C., Tommasi, F. and De Gara, L. (2002). Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco bright-yellow 2 cells. *Plant Physiol.* **130**, 698-708.

Desikan, R., Reynolds, A., Hancock, J. T. and Neill, S. J. (1998). Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in Arabidopsis suspension cultures. *Biochem. J.* **330**, 115-120.

Dong, H., Delaney, T. P., Bauer, D. W. and Beer, S. V. (1999). Harpin induces disease resistance in Arabidopsis through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *Plant J.* **20**, 207-215.

Dong, H. P., Peng, J. L., Bao, Z. L., Meng, X., Bonasera, J. M., Beer, S. V. and Dong, H. (2004). Downstream divergence of the ethylene signaling pathway for harpin-stimulated Arabidopsis growth and insect defense. *Plant Physiol.* **136**, 3628-3638.

Dong, H. P., Yu, H. Q., Bao, Z. L., Guo, X. J., Peng, J. L., Yao, Z. and Dong, H. (2005). The *ABI2*-dependent abscisic acid signalling controls HrpN-induced drought tolerance in Arabidopsis. *Planta* **221**, 313-327.

Dong, H. S. and Beer, S. V. (2000). Riboflavin induces disease resistance in plants by activating a novel signal transduction pathway. *Phytopathology* **90**, 801-811.

Dorey, S., Kopp, M., Geoffroy, P., Fritig, B. and Kauffmann, S. (1999). Hydrogen peroxide from the oxidative burst is neither necessary nor sufficient for hypersensitive cell death induction, phenylalanine ammonia lyase stimulation, salicylic acid accumulation, or scopoletin consumption in cultured tobacco cells treated with elicitin. *Plant Physiol.* **121**, 163-172.

Esau, K. (1965). *Plant Anatomy*, 2nd edn. New York: Wiley.

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J. M., Kircher, S. et al. (2008). Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. *Nature* **451**, 475-479.

Fisher, A. B. (2009). Redox signaling across cell membranes. *Antioxid. Redox Signal.* **11**, 1349-1356.

Fleischmann, F., Koehl, J., Portz, R., Beltrame, A. B. and Osswald, W. (2005). Physiological changes of *Fagus sylvatica* seedlings infected with *Phytophthora citricola* and the contribution of its elicitin "citricolin" to pathogenesis. *Plant Biol.* **7**, 650-658.

Ge, X. C., Li, G. J., Wang, S. B., Zhu, H. F., Zhu, T., Wang, X. and Xia, Y. J. (2007). AtNUDT7, a negative regulator of basal immunity in Arabidopsis, modulates two distinct defense response pathways and is involved in maintaining redox homeostasis. *Plant Physiol.* **145**, 204-215.

Glover, B. J., Perez-Rodriguez, M. and Martin, C. (1998). Development of several epidermal cell types can be specified by the same MYB2 related plant transcription factor. *Development* **125**, 3497-3508.

Huet, J. C., Salle-Tourne, M. and Pernollet, J. C. (1994). Amino acid sequence and toxicity of the alpha elicitin secreted with ubiquitin by *Phytophthora infestans*. *Mol. Plant Microbe Interact.* **7**, 302-304.

Huet, J. C., Le Caer, J. P., Nespoulous, C. and Pernollet, J. C. (1995). The relationships between the toxicity and the primary and secondary structures of elicitinlike protein elicitors secreted by the phytopathogenic fungus *Pythium vexans*. *Mol. Plant Microbe Interact.* **8**, 302-310.

Huitema, E., Vleeshouwers, V. G., Cakir, C., Kamoun, S. and Govers, F. (2005). Differences in intensity and specificity of hypersensitive response induction in *Nicotiana* spp. by INF1, INF2A, and INF2B of *Phytophthora infestans*. *Mol. Plant Microbe Interact.* **18**, 183-193.

Jabs, T., Dietrich, R. A. and Dangl, J. L. (1996). Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* **273,** 1853-1856.

Jiang, R. H. Y., Tyler, B. M., Whisson, S. C., Hardham, A. R. and Govers, F. (2006). Ancient origin of elicitin gene clusters in *Phytophthora* genomes. *Mol. Biol. Evol.* **23**, 338-351.

Johnson, H. B. (1975). Plant pubescence: an ecological perspective. *Bot. Rev.* **41**, 233-258.

Kamoun, S. (2001). Nonhost resistance to *Phytophthora*: novel prospects for a classical problem. *Curr. Opin. Plant Biol.* **4**, 295-300.

Kamoun, S., Klucher, K. M., Coffey, M. D. and Tyler, B. M. (1993). A gene encoding a host-specific elicitor protein of *Phytophthora parasitica*. *Mol. Plant Microbe Interact.* **6**, 573-581.

Kamoun, S., van West, P., de Jong, A. J., de Groot, K. E., Vleeshouwers,V. G. and Govers, F. (1997). A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Mol. Plant Microbe Interact.* **10**, 13-20.

Kamoun, S., van West, P., Vleeshouwers, V. G., de Groot, K. E. and Govers, F. (1998). Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. *Plant Cell* **10**, 1413-1426.

Kanneganti, T. D., Huitema, E., Cakir, C. and Kamoun, S. (2006). Synergistic interactions of the plant cell death pathways induced by *Phytophthora infestans* Nepl-like protein PiNPP1.1 and INF1 elicitin. *Mol. Plant Microbe Interact.* **19**, 854-863.

Kanzaki, H., Saitoh, H., Takahashi, Y., Berberich, T., Ito, A., Kamoun, S. and Terauchi, R. (2008). NbLRK1, a lectin-like receptor kinase protein of *Nicotiana benthamiana*, interacts with *Phytophthora infestans* INF1 elicitin and mediates INF1-induced cell death. *Planta* **228**, 977-987.

Karabourniotis, G., Papadopoulos, K., Papamarkou, M. and Manetas, Y. (1992). Ultraviolet-B radiation absorbing capacity of leaf hairs. *Physiol. Plantarum* **86**, 414-418.

Koehl, J., Djulic, A., Kirner, V., Nguyen, T. T. and Heiser, I. (2007). Ethylene is required for elicitin-induced oxidative burst but not for cell death induction in tobacco cell suspension cultures. *J. Plant Physiol.* **164**, 1555-1563.

Lebrun-Garcia, A., Ouaked, F., Chiltz, A. and Pugin, A. (1998). Activation of MAPK homologues by elicitors in tobacco cells. *Plant J.* **15**, 773-781.

Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R. and Pugin, A. (2002). Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell* **14**, 2627-2641.

Lequeu, J., Simon-Plas, F., Fromentin, J., Etienne, P., Petitot, A. S., Blein, J. P. and Suty, L. (2005). Proteasome comprising a β1 inducible subunit acts as a negative regulator of NADPH oxidase during elicitation of plant defense reactions. *FEBS Lett.* **21**, 4879-4886.

Liang, Y., Liu, A. and Dong, H. (2004). Elicitins and plant disease resistance (in Chinese with English abstract). *Microbiology* **31**, 134-137.

Liang, Y. C., Liu, A. X., Wang, J. S., Zhang, T. Y. and Dong, H. (2007). Microscopic hypersensitive response and expression of defense response induced by the proteinaceous elicitor elicitin-parasiticein (in Chinese with English abstract). *Acta Phytopathol. Sinica* **37**, 610-615.

Liu, F., Liu, H., Jia, Q., Wu, X., Guo, X., Zhang, S., Song, F. and Dong, H. (2006). The internal glycine-rich motif and cysteine suppress several effects of $HpaG_{Xooc}$ in plants. *Phytopathology* **96**, 1052-1059.

Lochman, J., Kasparovsky, T., Damborsky, J., Osman, H., Marais, A., Chaloupkova, R., Ponchet, M., Blein, J. P. and Mikes, V. (2005). Construction of cryptogein mutants, a proteinaceous elicitor from *Phytophthora*, with altered abilities to induce a defense reaction in tobacco cells. *Biochemistry* **44**, 6565-6572.

Loughner, R., Goldman, K., Loeb, G. and Nyrop, J. (2008). Influence of leaf trichomes on predatory mite (*Typhlodromus pyri*) abundance in grape varieties. *Exp. Appl. Acarol.* **45**, 111-122.

Mauricio, R. (1998). Costs of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. *Am. Nat.* **151**, 20-28.

Mikes, V., Milat, M., Ponchet, M., Panabieres, F., Ricci, P. and Blein, J. P. (1998). Elicitins, proteinaceous elicitors of plant defense, are a new class of sterol carrier proteins. *Biochem. Biophys. Res. Commun.* **245**, 133-139.

Morohashi, K., Zhao, M. Z., Yang, M. L., Read, B., Lloyd, A., Lamb, R. and Grotewold, E. (2007). Participation of the Arabidopsis bHLH factor GL3 in trichome initiation regulatory events. *Plant Physiol.* **145**, 736-746.

Mouton-Perronnet, F., Bruneteau, M., Denoroy, L., Bouliteau, F. P., Ricci, P., Bonnet, P. H. and Michel, G. (1995). Elicitin produced by an isolate of *Phytophthora parasitica* pathogenic to tobacco. *Phytochemistry* **38**, 41-44.

Osman, H., Mikes, V., Milat, M. L., Ponchet, M., Marion, D., Prange, T., Maume, B. F., Vauthrin, S. and Blein, J. P. (2001). Fatty acids bind to the fungal elicitor cryptogein and compete with sterols. *FEBS Lett.* **489**, 55-58.

Peng, J., Dong, H., Dong, H, P., Delaney, T. P., Bonasera, B. M. and Beer, S. V. (2003). Harpin-elicited hypersensitive cell death and pathogen resistance requires the *NDR1* and *EDS1* genes. *Physiol. Mol. Plant Pathol.* **62**, 317-326.

Peng, J., Bao, Z., Ren, H., Wang, J. and Dong, H. (2004). Expression of $harpin_{Xoo}$ in transgenic tobacco induces pathogen defense in the absence of hypersensitive response. *Phytopathology* **94**, 1048-1055.

Ponchet, M., Panabieres, F., Milat, M. L., Mikes, V., Montillet, J. L., Suty, L., Triantaphylides, C., Tirilly, Y. and Blein, J. P. (1999). Are elicitins cryptograms in plant-Oomycete communications? *Cell Mol. Life Sci.* **56**, 1020-1047.

Pugin, A., Frachisse, J. M., Tavernier, E., Bligny, R., Gout, E., Douce, R. and Guern, J. (1997). Early events induced by the elicitor cryptogein in tobacco cells: involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway. *Plant Cell* **9**, 2077-2091.

Quan, L. J., Zhang, B., Shi, W. W. and Li, H. Y. (2008). Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *J. Integr. Plant Biol.* **50**, 2-18.

Ren, H. Y., Gu, G. Y., Long, J. Y., Qian, J., Wu, T. Q., Song, T., Zhang, S. J., Chen, Z. and Dong, H. (2006a). Combinative effects of a bacterial type-III effector and a biocontrol bacterium on rice growth and disease resistance. *J. Biosci.* **31**, 617-627.

Ren, H., Song, T., Wu, T., Sun, L., Liu, Y., Yang, F., Chen, Z. and Dong, H. (2006b). Effects of a biocontrol bacterium on growth and defence of transgenic rice plants expressing a bacterial type-III effector. *Ann. Microbiol.* **56**, 281-287.

Ricci, P., Trentin, F., Bonnet, P., Venard, P., Mouton-Perronnet, F. and Bruneteau, M. (1992). Differential production of parasiticein, an elicitor of necrosis and resistance in tobacco, by isolates of *Phytophthora parasitica*. *Plant Pathol.* **41**, 298-307.

Riganti, C., Gazzano, E., Polimeni, M., Costamagna, C., Bosia, A. and Ghigo, D. (2004). Diphenyleneiodonium inhibits the cell redox metabolism and induces oxidative stress. *J. Biol. Biochem.* **279**, 47726-47731.

Roy, B. A., Stanton, M. L. and Eppley, S. M. (1999). Effects of environmental stress on leaf hair density and consequences for selection. *J. Evol. Biol.* **12**, 1089-1103.

Rusterucci, C., Stallaert, V., Milat, M. L., Pugin, A., Ricci, P. and Blein, J. P. (1996). Relationship between active oxygen species, lipid peroxidation, necrosis, and phytoalexin production induced by elicitins in *Nicotiana*. *Plant Physiol.* **111**, 885-891.

Rusterucci, C., Montillet, J. L., Agnel, J. P., Battesti, C., Alonso, B., Knoll, A., Bessoule, J. J., Etienne, P., Suty, L., Blein, J. P. et al. (1999). Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. *J. Biol. Chem.* **274**, 36446-36455.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y. and Hunt, M. D. (1996). Systemic acquired resistance. *Plant Cell* **8**, 1809-1819.

Sagi, M. and Fluhr, R. (2006). Production of reactive oxygen species by plant NADPH oxidases. *Plant Physiol.* **141**, 336-340.

Sasabe, M., Takeuchi, K., Kamoun, S., Ichinose, Y., Govers, F., Toyoda, K., Shiraishi, T. and Yamada, T. (2000). Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture. *Eur. J. Biochem.* **267**, 5005-5013.

Schagger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulphate-polyacrylamid gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368-379.

Skaltsa, H., Verykokidou, E., Harvala, C., Karabourniotis, G. and Manetas, Y. (1994). UV-B protective potential and flavonoid content of leaf hairs of *Quercus ilex*. *Phytochemistry* **37**, 987-990.

Szymanski, D. B., Lloyd, A. M. and Marks, M. D. (2000). Progress in the molecular genetic analysis of trichome initiation and morphogenesis in Arabidopsis. *Trends Plant Sci.* **5**, 1360-1385.

Takemoto, D., Hardham, A. R. and Jones, D. A. (2005). Differences in cell death induction by Phytophthora elicitins are determined by signal components downstream of MAP kinase kinase in different species of Nicotiana and cultivars of *Brassica rapa* and *Raphanus sativus*. *Plant Physiol.* **138**, 1491-1504.

Tanaka, N., Che, F. S., Watanabe, N., Fujiwara, S., Takayama, S. and Isogai, A. (2003). Flagellin from an incompatible strain of *Acidovorax avenae* mediates H_2O_2 generation accompanying hypersensitive cell death and expression of PAL, Cht-1, and PBZ1, but not of Lox in rice. *Mol. Plant Microbe Interact.* **16**, 422-428.

Tao, X. and Zhou, X. (2004). A modified viral satellite DNA that suppresses gene expression in plants. *Plant J.* **38**, 850-860.

Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D. B. (1997). Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* **11**, 1187-1194.

Torres, M. A., Jones, J. D. G. and Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* **141**, 373-378.

Traw, M. B. and Bergelson, J. (2003). Interactive effects of jasmonic acid, salicylic acid, and gibberellin on induction of trichomes in Arabidopsis. *Plant Physiol.* **133**, 1367-1375.

Traw, M. B. and Feeny, P. (2008). Glucosinolates and trichomes track tissue value in two sympatric mustards. *Ecology* **89**, 763-772.

Trouvelot, S., Varnier, A. L., Allegre, M., Mercier, L., Baillieul, F., Arnould, C., Gianinazzi-Pearson, V., Klarzynski, O., Joubert, J. M., Pugin, A. et al. (2008). A β-1,3 glucan sulfate induces resistance in grapevine against *Plasmopara viticola* through priming of defense responses, including HR-like cell death. *Mol. Plant Microbe Interact.* **21**, 232-243.

Uphof, J. C. and Hummel, K. (1962). *Plant Hairs.* Berlin-Nikolassee: Gebrüder Borntraeger.

Van Breusegem, F. and Dat, J. F. (2006). Reactive oxygen species in plant cell death. *Plant Physiol.* **141**, 384-390.

Vauthrin, S., Mikes, V., Milat, M. L., Ponchet, M., Maume, B., Osman, H. and Blein, J. P. (1999). Elicitins trap and transfer sterols from micelles, liposomes and plant plasma membranes. *Biochim. Biophys. Acta* **1419**, 335-342.

Wagner, G. J. (1991). Secreting glandular trichomes: more than just hairs. *Plant Physiol.* **96**, 675-679.

Walker, A. R., Davison, P. A., Bolognesi-Winfield, A. C., James, C. M., Srinivasan, N., Blundell, T. L., Esch, J. J., Marks, M. D. and Gray, J. C. (1999). The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. *Plant Cell* **11**, 1337-1349.

Wei, Z. M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A. and Beer, S. V. (1992). Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**, 85-88.

Weigel, D. and Glazebrook, J. (2002). *Arabidopsis*: *A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Yoshioka, H., Numata, N., Nakajima, K., Katou, S., Kawakita, K., Rowland, O., Jones, J. D. and Doke, N. (2003). *Nicotiana benthamiana* gp^{91phox} homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. *Plant Cell* **15**, 706-718.

Yu, L. M. (1995). Elicitins from *Phytophthora* and basic resistance in tobacco. *Proc. Natl. Acad. Sci. USA* **92**, 4088-4094.

Zhang, C. L., Bao, Z. L., Liang, Y. C., Yang, X., Wu, X. J., Hong, X. and Dong, H. (2007). Abscisic acid mediates Arabidopsis drought tolerance induced by $HrpN_{Ea}$ in the absence of ethylene signaling. *Plant Mol. Biol. Rep.* **25**, 98-114.

Zhang, S. J., Yang, X., Sun, M. W., Sun, F., Deng, S. and Dong, H. (2008). Riboflavin-induced priming for pathogen defense in *Arabidopsis thaliana*. *J. Integr. Plant Biol.* **51**, 167-174.

Zhao, M., Morohashi, K., Hatlestad, G., Grotewold, E. and Lloyd, A. (2008). The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci. *Development* **135**, 1991-1999.