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A *PAL1* gene promoter–green fluorescent protein reporter system to analyse defence responses in live cells of *Arabidopsis thaliana*

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Abstract

Arabidopsis thaliana ecotype Columbia-0 was transformed with a green fluorescent protein (GFP) gene under control of a phenylalanine ammonia-lyase (PAL) promoter. PAL is a key enzyme of the phenylpropanoid pathway and is induced to high levels during plant stress. Constitutive expression of *PAL1* promoter-controlled GFP occurred in vascular tissues within stems, leaves and roots and in developing flowers. *PAL1* promoter–GFP expression was examined in leaves of transgenic plants subjected to an abiotic elicitor, mechanical wounding or to inoculation with the pathogens *Pseudomonas syringae* pv. *tomato* or *Peronospora parasitica.* Wounding of leaves and treatment with an abiotic elicitor and compatible interactions produced low to moderate levels of GFP. However, in incompatible interactions there were high levels of GFP produced. In incompatible interactions, the intensity of GFP fluorescence was similar to that produced in transgenic plants expressing GFP driven by the CaMV promoter. The bright green fluorescence produced in live cells and tissues was readily visualised using conventional fluorescence microscopy and was quantified using spectroflourometry. This is the first report of the use of GFP as a reporter of defence gene activation against pathogens. It has several advantages over other reporter genes including real time analysis of gene expression and visualisation of defence gene activation in a non-invasive manner.

Introduction

The enzyme, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), provides the first committed step towards the biosynthesis of phenylpropanoids – a large group of plant metabolites that have primary roles in development and protection against environmental stresses (Dixon and Paiva, 1995; Weisshaar and Jenkins, 1998). The compounds produced via the phenylpropanoid pathway include flavonoid derivatives such as flower pigments, components of structural polymers including lignin and suberin, intercellular signalling molecules, UV protection components and antimicrobial phytoalexins. Transcription of PAL genes can be induced by a range of biotic and abiotic factors including wounding, UV-light, infection by pathogens or exposure to pathogen-derived elicitors and by the salts of heavy metals (Kervinen et al., 1998; Levee and Seguin, 2001; Smith et al., 2001).

Phenylalanine ammonia-lyase genes have been characterised in a number of species and usually comprise of families of between two and six members. For example, the PAL gene family in *Arabidopsis thaliana* has three members designated *PAL1*, *PAL2* and *PAL3*. The spatial pattern of *PAL1* and *PAL2* gene expression is similar while *PAL1* is more abundant; *PAL3* is produced at much lower levels (Wanner et al., 1995). Many studies of PAL gene regulation and expression have been conducted using Northern hybridisation analysis, a sensitive technique but one that cannot provide accurate spatial data of gene activation especially at the level of the single cell in live tissues. Another approach for examining PAL gene expression has been to use specific reporter molecules that

are induced upon activation of the PAL promoter. For example, β-glucuronidase (GUS) or luciferase reporter enzymes have been used to examine PAL gene expression in transgenic tobacco (Gray-Mitsumune et al., 1999; Liang et al., 1989), bean (Bevan et al., 1989) and *A. thaliana* (Giacomin and Szalay, 1996). While both reporter enzymes allow study of PAL gene activation, GUS activity can only be assessed in killed cells and luciferase detection requires a photon counting camera and addition of a substrate that may not penetrate through cell walls (Millar et al., 1992).

In this study, we describe a system to examine cellular changes in *PAL1* gene expression using a reporter, the green fluorescent protein (GFP), from the jellyfish *Aequorea victoria*, that can be visualised easily within live tissues using conventional fluorescence microscopy. The soluble, modified GFP (smGFP) used was developed by Davis and Viestra (1998) and contains site directed mutations that provide correct splicing of the gene in *A. thaliana* and an improved fluorescence signal. GFP provides several advantages over other reporter genes as it is non-invasive, can be visualised in living cells, is non-toxic and requires only oxygen as a substrate (Davis and Viestra, 1998; Heim et al., 1994).

Green fluorescent protein is becoming more widely used as a reporter molecule to analyse structural, developmental and biochemical processes within plants. For example, GFP has been used for gene expression studies, analysis of cytoskeletal rearrangements, detection of enzyme activities and their localisation and as a specific organelle marker (Cutler et al., 2000; Marc et al., 1998; Niwa et al., 1999; Ro et al., 2001; Sidorov et al., 1999). The use of GFP to elucidate elements of the interactions between plants and pathogens has, however, been limited. GFP has primarily been used as a fluorescent marker to examine inter- and intracellular distribution of pathogens including viruses (Canto and Palukaitis, 1999; Cohen et al., 2000), pathogenic fungi and oomycetes. For the latter groups the tissue and cellular locations of *Ustilago maydis* in corn (Spellig et al., 1996), *Mycosphaerella* species in banana (Balint-Kurti et al., 2001), and *Phytophthora parasitica* var. *nicotianae* in tobacco (Bottin et al., 1999) have all used GFP as a marker.

Previous research that has used the PAL promoter linked to a reporter gene has concentrated on PAL gene expression during development and few have examined defence-related PAL gene expression. Mauch-Mani and Slusarenko (1996) have shown activation of the expression of *PAL1* in incompatible interactions of *A. thaliana* with *P. parasitica* using a GUS reporter, and Giacomin and Szalay (1996) have shown activation of *PAL1* in incompatible interactions of *A. thaliana* with *Pseudomonas syringae* pv. *tomato* using a luciferase (*lux F*) reporter. Both provided a preliminary analysis of pathogen induced *PAL1* promoter activity but did not fully investigate the spatial and temporal regulation of PAL in the defence response. This study demonstrates that responses of live leaf cells of *A. thaliana* to artificial wounding, application of an abiotic elicitor, and to inoculation with two commonly used pathogens, can be followed using the GFP reporter gene linked to a *PAL1* promoter. We also show that in Arabidopsis induction of the phenylpropanoid pathway is closely associated with restriction of pathogens during incompatibility.

Materials and methods

Plant material, media and bacterial strains

Arabidopsis thaliana (ecotype Columbia-0 (Col-0)) seeds were sterilised with 10% bleach containing 0.1% (v/v) Triton X-100 (BDH Chemicals, Australia) for several minutes and washed repeatedly with sterile distilled water. Seeds were sown on Murashige and Skoog (MS) (Sigma, USA) medium in Petri plates and vernalised at 4 °C in the dark for 4 days. After vernalisation, the plates were placed in a controlled environment room at a temperature of 21 °C with a 12 h light/dark cycle provided by 400 W high pressure sodium lights (Osram, Australia). After 10–14 days growth seedlings were removed from the MS plates and transferred to a soil mixture (Debco potting mixture, Australia) within 10 cm plastic pots.

For plasmid amplification, electroporation was used to transfer vectors into *Escherichia coli* strain DH5α (Invitrogen, Australia) that was grown at 37 °C on Luria-Bertani medium (Sambrook et al., 1989). *Ps. syringae* pv. *tomato* strains DC3000 (virulent with *A. thaliana* Col-0) and JL1065 (avirulent with *A. thaliana* ecotype Col-0) (Whalen et al., 1991) were grown at 30 °C on King's broth (King et al., 1954). Electrocompetent *Agrobacterium tumefaciens* LBA 4404 (Invitrogen, Australia) was grown on YM medium at 30 °C (Lin, 1994). Where required, the media were supplemented with antibiotics (Sigma, USA) at the following concentrations; 50 μg ml^{-1} kanamycin sulphate, 50 μg ml^{-1} rifampicin, 100 μg ml^{-1} ampicillin and 100 μg ml^{-1} streptomycin sulphate.

Peronospora parasitica isolates Hind 4 and Noks 1 were maintained on *A. thaliana* ecotype Wassilewskija (compatible interactions) and subcultured weekly (Mauch-Mani and Slusarenko, 1996). To induce infection and maintain pathogen growth, plants were grown within a small growth chamber (SEM, South Australia) under high humidity at 16 °C with a 12 h light/dark cycle.

Binary vector construction

Vectors were based on the plasmid, pBI121, constructed by Jefferson et al. (1987). The cauliflower mosaic virus (CaMV) promoter, GUS gene and nopaline synthase polyadenylation site (NOS-T) were removed from the vector with Hind III and Eco R1 restriction enzymes (Invitrogen, Australia). The gene for smGFP was derived from the plasmid psmGFP (Davis and Viestra, 1998), obtained from the Arabidopsis Resource Centre (OH, USA). The smGFP gene was contained between the CaMV promoter and the NOS-T region and to remove the whole fragment from the plasmid restriction enzymes Hind III and Eco R1 were used. The CaMV promoter/smGFP gene/NOS-T fragment was then ligated directly into the Hind III and Eco R1 sites of cut pBI121. The newly constructed vector was denoted pCMVGFP. Correct orientation of the ligated sequence was verified using a dideoxy chain termination method (Sambrook et al., 1989).

Polymerase chain reaction (PCR) was used to amplify the full length 1.8 kb *PAL1* promoter fragment from genomic DNA (gDNA) of *A. thaliana* (ecotype Col-0). Two primers, 5′-TAGAAGCTT TGACATAAATAGGAGGCTTTTGC (starting at −1715) and 5′-TAGGGATCCGAGGAGATTACAAG ATCTCTAAAAGAA (starting at +88) were designed using primer design software (Primer3 software, Whitehead Institute for Biomedical Research, USA). Primers included Hind III and Bam H1 restriction sites (underlined) for easy ligation and were used to amplify the entire promoter region. The fragment obtained was ligated into cut psmGFP. This plasmid was denoted psm*PAL1*GFP. The correct orientation of the ligated sequence was again verified using a dideoxy chain termination method (Sambrook et al., 1989). The binary vector pBI121 was again cut at the Hind III and Eco R1 restriction enzyme sites into which the *PAL1* promoter/smGFP/NOS-T fragment from psm*PAL1*GFP was ligated. This vector was denoted pPALGFP (Figure 1).

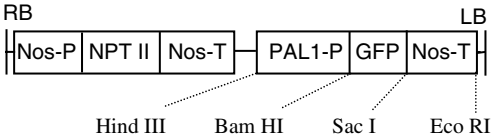

Figure 1. Structure of the T-DNA region of the binary vector pPALGFP. Right border (RB) region contains the kanamycin resistance gene, neomycin phosphotransferase II (NPT II), controlled by the nopaline synthase promoter (NOS-P), followed by the nopaline synthase polyadenylation site (NOS-T). Left border (LB) region shows the smGFP (GFP) gene under the control of the *PAL1* promoter (PAL1-P) and NOS-T. The positive control construct pCMVGFP contained the CaMV (CaMV 35S) in place of the *PAL1* promoter. Cleavage sites for the restriction enzymes used are also shown. Note that the diagram is not to scale.

Plant transformation

Arabidopsis thaliana was transformed using the floral dip technique (Clough and Bent, 1998). Briefly, plants of 5 weeks of age with inflorescence stalks of less than 10 cm were dipped in a suspension (OD_{600} 0.8) of *A. tumefaciens* LBA 4404 in 5% sucrose and 0.05% Silwett L-77 (Lehle Seeds, USA) for several seconds. Plants were grown to seed, the seeds were collected and screened for the insert by plating on MS plates with 50 μg ml^{-1} kanamycin. Germinated seedlings were transferred onto fresh MS-kanamycin plates for 7 days and then transferred to soil mixture in pots. Positive transgenic lines were grown to the T_3 generation with no change in phenotype.

Elicitor treatment, wounding and pathogen inoculation

For all treatments and inoculations, a single transgenic line, designated line 1, was used. Wounding of leaf tissue involved abrasion of the surface of leaves with a fine pipette tip. For treatment of plants with the abiotic elicitor a micropipette was used to place two 2-μl droplets of a mercuric chloride solution ($HgCl_2$, 100 μM in distilled water) onto the abaxial side of leaves of 4-week-old plants. Plants were returned to the standard growth conditions.

For inoculation of leaves with the isolates of *Ps. syringae* pv. *tomato*, cultures were grown overnight in King's broth and resuspended to provide a density of approximately 10^8 colony forming units ml^{-1} in 10 mM $MgCl_2$ (Dong et al., 1991). The adaxial side of attached leaves of *A. thaliana* were inoculated at a single site via syringe infiltration using a

1 ml plastic disposable syringe (without needle) that was held with the tip against the leaf surface. Bacterial suspension was gently forced into intercellular spaces over an area of 3 mm in radius by slightly depressing the syringe plunger (Thomma et al., 2001). Upon inoculation, plants were placed in sealed, clear plastic boxes and returned to the growth cabinet.

For inoculation of leaves with isolates of *P. parasitica* conidial suspensions (10^5 conidia ml^{-1} sterile tap water) were prepared as described by Mauch-Mani and Slusarenko (1994). Leaves of *A. thaliana* were inoculated on the abaxial surface by placement with a micropipette of four 2-μl droplets of suspension. Inoculated plants were placed in clear plastic boxes and returned to the growth cabinet.

Visualisation of GFP expression within elicitor-treated, wounded or inoculated leaf tissue

At various times after inoculation (every 3 h until 12, 18, 24, 48 h and then every 24 h for the next 3 days for *Ps. syringae*; every 24 h for 6 days for *P. parasitica*) or elicitor treatment (every hour until 6 h after treatment then every 6 h until 24 h) leaves were excised from plants using fine scissors and divided into 4 × 4 mm pieces. Single pieces were immediately mounted in distilled water on a microscope slide and covered with a coverslip. Expression of GFP was examined in leaf cells and tissues by viewing with a microscope (Axioskop 20, Carl Zeiss Pty Ltd, Australia) equipped with both UV (excitation wavelength 365 nm, emission 420 nm) and blue light (excitation 450–490 nm, emission 520 nm) filter sets for fluorescence optics. Images of tissues and individual cells were captured using a Spot RT digital camera (Spot Diagnostics, Australia) mounted on the microscope and were optimised using ImagePro Express software (Media Cybernetics, USA).

Spectrofluorometry of leaf extracts

Leaf extracts were prepared for spectrofluorometry by grinding 200 mg of leaf tissue from 4-week-old plants in liquid nitrogen with a mortar and pestle. The resulting powder was suspended in a protein extraction buffer (50 mM NaH_2PO_4, 10 mM Na_2 EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, pH 7) within a microfuge tube. The tube was vortexed vigorously and centrifuged for 5 min at 14 000*g*. The supernatant was transferred to a fresh tube and centrifuged again. Protein concentration was measured using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

Green fluorescent protein fluorescence was measured in the supernatant within a 3 ml cuvette by exposure to an excitation wavelength of 395 nm within a spectrofluorometer (Varian Eclipse, Melbourne, Australia). GFP has an emission peak at 507 nm therefore, the instrument was set to record an emission spectrum between 450 and 600 nm. Emission spectra were generated for each leaf extract and the fluorescence determined as relative fluorescence units (rfu) mg^{-1} protein.

Plant DNA isolation and analysis

Arabidopsis thaliana gDNA was isolated from plants that were 4–6 weeks old using a plant DNA extraction kit (DNeasy Plant System, Qiagen Pty Ltd, Australia). For use in dot blot and Southern analysis the concentration of DNA was determined spectrophotometrically. Dot blot analysis was used to detect the presence of T-DNA inserts in gDNA at concentrations of 200 ng and 2 μg. Southern blot analysis was used to determine T-DNA insert copy number using approximately 10 μg gDNA. The DNA probes that were used were constructed as previously described and labelled using an alkaline phosphatase direct labelling system (AlkPhos Direct, Amersham Pharmacia Biotech, Australia). Hybond Nylon membrane (Amersham Pharmacia Biotech, Australia) was used for all blotting applications and DNA was bound to the membrane by baking at 80 °C for 2 h. Presence of bands that hybridised with the probes were detected using a chemiluminescence assay (CDP-star reagent, Amersham Pharmacia Biotech, Australia) and chemiluminescence film to capture the signal.

Results

Transformation, screening and selection of PAL1 *promoter–GFP (PALGFP) and CaMV promoter–GFP (CMVGFP) lines of* A. thaliana

Arabidopsis thaliana Col-0 was transformed using *A. tumefaciens* containing the binary vectors pPALGFP (Figure 1) or pCMVGFP. Following transformation, seedlings of *A. thaliana* were screened for growth

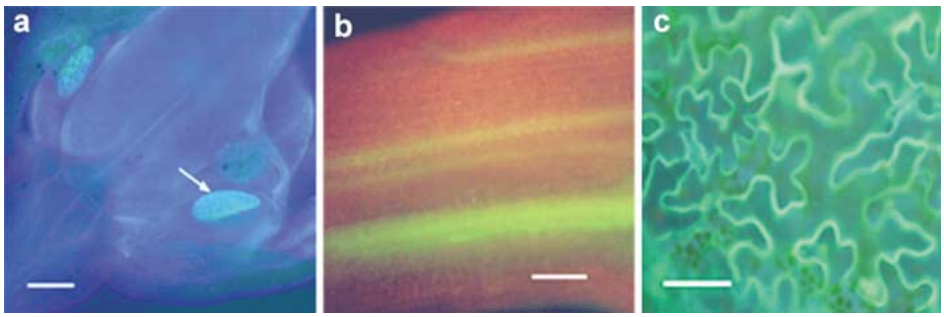

Figure 2. GFP expression in transgenic *A. thaliana.* Figures (a) and (b) show the expression of GFP in transgenic plants that contained the insert from pPALGFP and, (c) the insert from pCMVGFP. (a) Ultraviolet light-induced fluorescence of GFP within a longitudinal section through the flower. Green fluorescence is associated with developing anthers (arrow). Scale bar denotes 150 μm. (b) Blue light-induced fluorescence of GFP within the vascular tissue of an inflorescence stalk. GFP appears yellow-green against the red background that is the product of chloroplast autofluorescence. Scale bar denotes 100 μm. (c) Ultraviolet light-induced fluorescence of GFP within leaf epidermal cells. All cells express GFP to a high level. Scale bar denotes 50 μm.

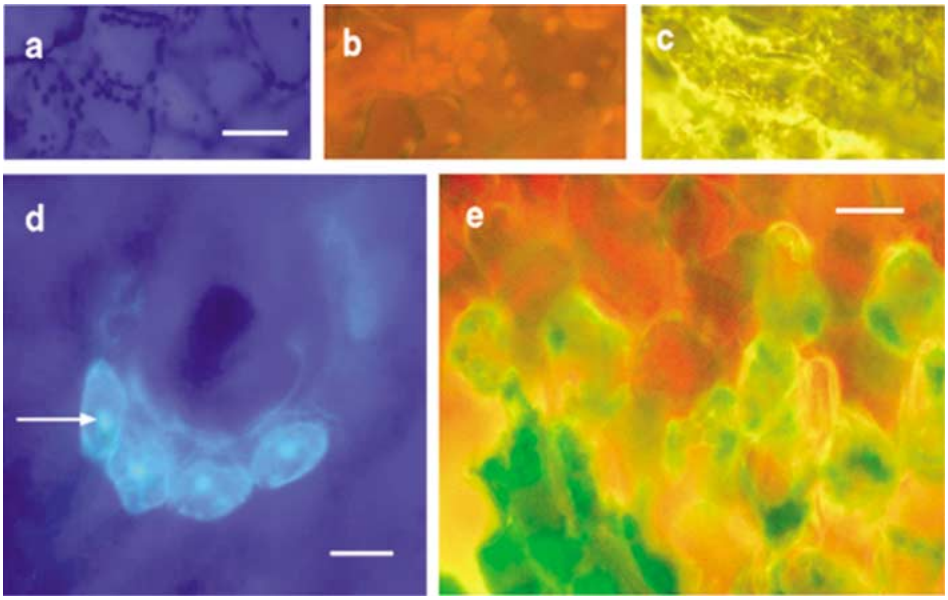

Figure 3. Blue light and ultraviolet light-induced autofluorescence of *A. thaliana* leaf tissues and the production of GFP following wounding or application of mercuric chloride. (a) Ultraviolet light-induced fluorescence of leaf cells. All cells have a deep blue colour. Scale bar represents 50 μm. (b) Blue light-induced fluorescence of leaf cells showing the bright red autofluorescence of chloroplasts within cells. Scale as in (a). (c) Blue light-induced yellow autofluorescence of senescent cells. Scale as in (a). (d) Ultraviolet light-induced fluorescence of GFP within trichome basal cells 24 h after damage (bending) during treatment. Note the expression of GFP within the nucleus (arrow) and surrounding cytoplasmic strands. Scale bar represents 50 μm. (e) Blue light-induced fluorescence of GFP within leaf cells 6 h after the application of 100 μM $HgCl_2$. The image was taken from the edge of the treatment droplet. There is a mixture of fluorescent colours in the figure: green = GFP fluorescence; yellow = autofluorescence of affected cells; red = chloroplast autofluorescence. Scale bar represents 50 μm.

on selective MS medium containing kanamycin. Six kanamycin resistant PALGFP lines and four kanamycin resistant CMVGFP lines were obtained at a transformation rate of 0.16% and 0.2%, respectively. The presence of the GFP gene in transformed lines was confirmed using fluorescence microscopy to detect ultraviolet light- and blue light-induced fluorescence of GFP in cells and tissues (Figure 2a–c). A preliminary

screen that used the PCR to amplify a 684 bp fragment of the GFP gene in gDNA samples was also used as additional confirmation (data not shown).

Expression and distribution of GFP in planta

The PALGFP lines showed GFP expression in vascular tissues and floral components such as the anthers (Figure 2a and b). Root tissue showed a low to medium level of GFP expression within vascular tissues whereas leaf epidermal tissue displayed no discernable GFP expression. The CMVGFP lines expressed GFP in root, stem and leaf tissues due to control by the strong constitutive CaMV 35s promoter (Figure 2c).

Polymerase chain reaction screening of kanamycin resistant lines showed that of the transformed lines one PALGFP line did not contain the GFP gene. All other lines contained the GFP gene and provided GFP expression in similar patterns. All transgenic lines were phenotypically identical except one line of CMVGFP that had stunted growth and altered leaf and flower shape. This line still showed GFP expression that was similar to other lines but was not used in further experiments.

Dot blot and Southern analysis of transformed lines

Genomic DNA from two PALGFP lines was tested for presence of the GFP gene using dot blot analysis and a GFP gene fragment as a probe. Both lines tested positive for the presence of the GFP gene. Southern hybridisation analysis was then used to determine the number of copies of the insert in each line. Using a GFP gene fragment as a probe, PALGFP line 1 was revealed to contain one T-DNA copy, while PALGFP line 2 contained two copies. An additional Southern hybridisation was conducted using a fragment from the *PAL1* promoter as a probe that produced an identical banding pattern to the original Southern hybridisation. There was also an additional band present that was identified as the endogenous *A. thaliana PAL1* promoter by comparison with a Southern analysis of wild type *A. thaliana* Col-0 gDNA.

Induction of GFP expression in PALGFP lines following treatment with $HgCl_2$ or wounding

Untreated control leaf tissues that were exposed to ultrviolet light showed a dull blue fluorescence (Figure 3a). Exposure to blue light induced bright red autofluorescence in cells that contained chloroplasts (Figure 3b), and yellow autofluorescence in senescent cells (Figure 3c). Mechanical wounding initiated GFP accumulation around the wound site within 2–3 h (Table 1) and damage to trichomes resulted in GFP production in associated basal cells (Figure 3d). After treatment with mercuric chloride GFP was produced within 2–3 h in many cells located beneath the treatment droplet (Figure 3e).

Response of PALGFP lines to inoculation with avirulent and virulent isolates of P. parasitica *and* Ps. syringae *pv.* tomato

To ensure that the typical compatible and incompatible interactions were produced in transgenic lines, *P. parasitica* and *Ps. syringae* pv. *tomato* were inoculated onto leaves and interactions observed over time. Leaves that were inoculated with the avirulent isolate (Hind 4) had the typically restricted hypersensitive lesions as revealed by lactophenol-trypan blue staining (Figure 4a). Inoculation of leaves of the transgenic *A. thaliana* with a virulent isolate of *P. parasitica* (Noks 1) resulted in a typical compatible interaction with spread of the pathogen throughout leaf tissues and eventual sporulation on the leaf surface by 120 h (Figure 4b). In the incompatible interaction with *P. parasitica* (Hind 4) GFP was detected by 72 h after inoculation and high level expression was found at 120 h after inoculation (Table 1). High level expression of GFP was maintained until 168 h after inoculation and then declined. Cells that produced GFP formed a distinct 'halo' around individual lesions (Figure 4c, d and g). Single cells in contact with *P. parasitica* spores were also shown to produce GFP, while lower GFP levels were observed in adjoining cells (Figure 4e and f). In the compatible interaction with *P. parasitica* (Noks 1) there was only low level induction of GFP expression initiated 120–144 h after inoculation (Table 1, Figure 4h).

Following inoculation of leaves by the virulent isolate of *Ps. syringae* pv. *tomato* (DC3000) a compatible interaction was evident by 72 h as slight chlorosis and malformation of leaf tissues around the infiltration site (data not shown). In contrast, in incompatible interactions with the avirulent isolate of *Ps. syringae* pv. *tomato* (JL1065), groups of cells that had responded hypersensitively were evident at 48–72 h after inoculation (data not shown). A time

Table 1. Initiation and intensity of GFP in leaves of transgenic *A. thaliana* (PALGFP line 1) following wounding, treatment with $HgCl_2$ or inoculation with virulent or avirulent isolates of *Ps. syringae* and *P. parasitica*

Treatment	Reaction to pathogen	Initial GFP induction (h)	Time to maximum GFP intensity (h)	Relative maximum intensity level[1]
Control	NT[2]	ND	ND	−
Mechanical wounding	NT	2–3	12	+
100 μM $HgCl_2$	NT	2–3	6	++
Pseudomonas syringae JL1065	Incompatible	12–18	48	+++
Pseudomonas syringae DC3000	Compatible	ND	ND	−
Peronospora parasitica Hind 4	Incompatible	72	120	+++
Peronospora parasitica Noks 1	Compatible	120	144	+

[1]Relative intensity of GFP was determined by microscopic observation with reference to the level observed upon maximal expression of the pCMVGFP insert in leaf tissues. Where: −, No GFP expression; +, low level GFP expression; ++, intermediate GFP expression; +++, high level GFP expression (equivalent to CMVGFP expression).
[2]NT, not tested; ND, not detected.

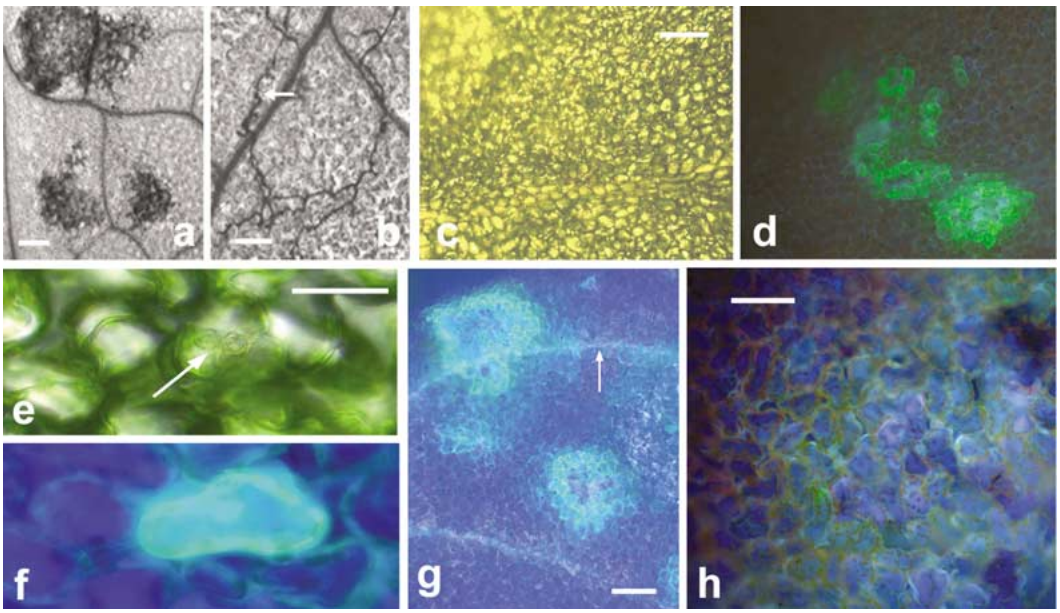

Figure 4. Pathogen spread and GFP expression in transgenic *A. thaliana* (PALGFP) after inoculation with avirulent or virulent isolates of *P. parasitica*. (a) Whole mount of a leaf stained with lactophenol-trypan blue 168 h after inoculation with avirulent *P. parasitica* Hind 4 showing darkly stained hypersensitive lesions. Scale bar represents 200 μm. (b) Whole mount of lactophenol-trypan blue stained leaf 168 h after inoculation with virulent *P. parasitica* Noks 1. Hyphae (arrow) are clearly visible penetrating through the leaf tissue. Scale bar represents 200 μm. (c) Whole mount of unstained leaf 120 h after inoculation with an avirulent *P. parasitica* (Hind 4) under white light and (d) ultraviolet light. The image in (d) shows GFP expression confined to a 'halo' around inoculated sites that have reacted hypersensitively. Scale bar represents 200 μm. (e) Phase contrast image of a group of leaf cells that show two *P. parasitica* (Hind 4) spores on the surface (arrow). Scale bar represents 50 μm. (f) Ultraviolet light-induced fluorescence of GFP in cells shown in (e). Note the intense fluorescence of the cell directly below the spores shown in (e). (g) GFP expression within unaffected vascular leaf tissue (arrow) and in hypersensitive tissue following inoculation with an avirulent isolate of *P. parasitica* (Hind 4) 168 h post inoculation. Note that the image has been overexposed to reveal the vascular location of GFP. Scale bar represents 200 μm. (h) Low level fluorescence of GFP induced by ultraviolet light in the interaction with the virulent *P. parasitica* (Noks 1) 144 h post inoculation. Scale bar represents 100 μm.

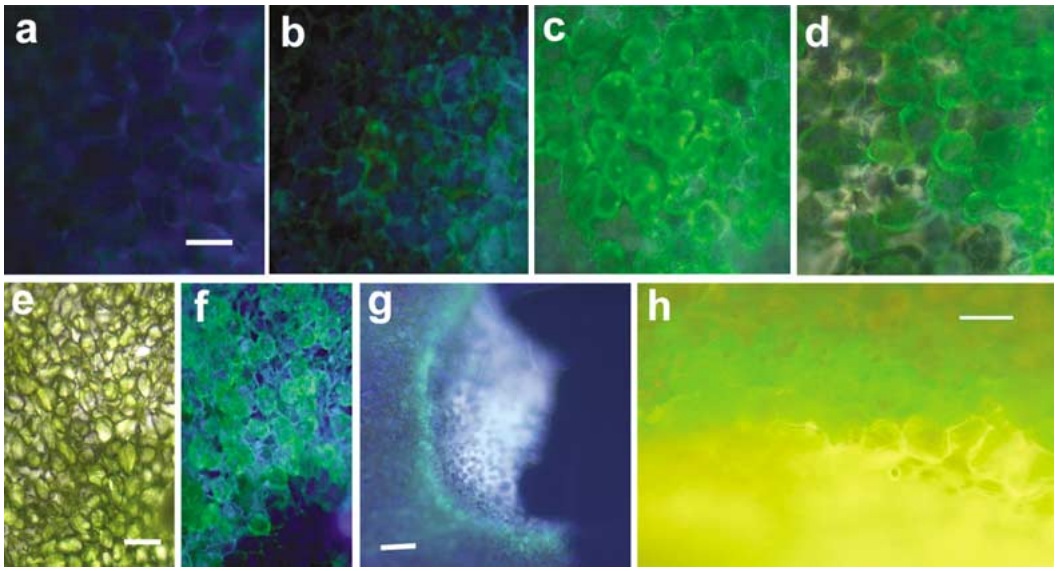

Figure 5. Time course of GFP production in transgenic *A. thaliana* (line 1) after inoculation with avirulent or virulent isolates of *Ps. syringae* pv. *tomato*. (a)–(d) show a time course of GFP production following inoculation of leaves with *Ps. syringae* pv. *tomato* (JL1065) (a) 0 h, (b) 18 h, (e) 48 h and (d) 96 h after inoculation. Scale bar represents 50 μm. (e) The virulent interaction with *Ps. syringae* pv. *tomato* (DC3000) 48 h after inoculation showing relatively healthy leaf cells under white light illumination. Scale bar represents 100 μm. (f) Ultraviolet light exposure of the same region in (e) reveals GFP production at the inoculation site. Figures (g) and (h) show GFP production at the lesion margin 72 h after inoculation with avirulent *Ps. syringae* pv. *tomato* (JL1065) under ultraviolet (g) and blue light (h) excitation. Under blue light, bright yellow autofluorescence was found to occur in necrotic cells at the centre of the lesion. Scale bars represents 200 and 50 μm, respectively.

course study of the induction of GFP within cells during the incompatible interaction of *Ps. syringae* pv. *tomato* (JL1065) showed that the intensity of GFP fluorescence in challenged leaf cells increased with time after inoculation (Figure 5a–d). GFP was detected 12–18 h after inoculation and reached maximal levels of expression at 48 h and corresponded closely to the development of hypersensitive lesions (Figure 5e and f). The total amount of GFP fluorescence at lesion sites remained at the maximal level until 72–96 h after inoculation and then declined. As in the interaction with the avirulent isolate of *P. parasitica*, during later stages of the interaction a 'halo' of cells with high levels of GFP fluorescence was observed. The 'halo' formed around the periphery of lesions within which a central zone of dead and autofluorescent cells was observed (Figure 5g and h). GFP production was not detected in leaves at any time point after inoculation with the virulent *Ps. syringae* pv. *tomato* (DC3000) (Table 1).

Spectrofluorometric analysis

Protein extracts from leaf tissue of transgenic and non-transgenic lines of *A. thaliana* were examined by spectrofluorometry for the presence of GFP. A high level of fluorescence was found in extracts from CMVGFP line 1 with a maximum at 507 nm (76.4 ± 4.2 rfu mg^{-1} protein) (Figure 6a), in accordance with the high levels of GFP visualised under UV light illumination in this constitutively controlled line. A smaller peak at 507 nm corresponding to 32.5 ± 4.8 rfu mg^{-1} protein was produced from leaf tissue extracts derived from PALGFP line 1 and for wild type non-transgenic plants 24.4 ± 0.9 rfu mg^{-1} protein.

To test the sensitivity of this semi-quantitative technique for the detection of PAL gene activation during avirulent pathogen challenge, GFP levels were measured in leaf extracts 5 days after inoculation of PALGFP line 1 with *P. parasitica* (Hind 4) (Figure 6b). There was a high level of fluorescence at 507 nm (73.1 rfu ± 11.1 mg^{-1} protein) in leaf extracts from inoculated plants compared with non-inoculated controls (35 ± 5.9 rfu mg^{-1} protein). Non-transgenic inoculated plants produced fluorescence equivalent to 31.6 ± 3.8 rfu mg^{-1} protein. In non-transgenic plants a low level increase in fluorescence was found that can likely be attributed to the production of autofluorescent compounds during the interaction.

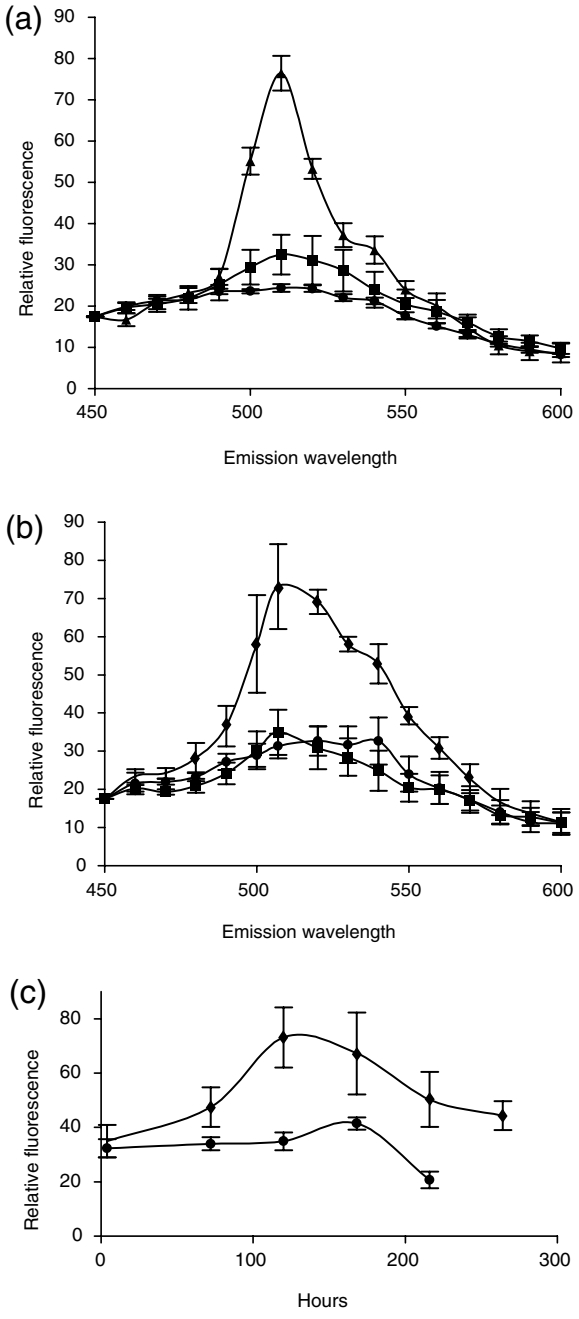

Figure 6. Relative fluorescence of GFP in extracts from transgenic and non-transgenic *A. thaliana* leaf tissues. (a) The relative fluorescence of leaf extracts from CMVGFP (□) and PALGFP (■) and Col-0 (●). Bars represent standard error of the mean ($n = 3$). (b) The relative fluorescence of leaf extracts from PALGFP (○) 120 h after inoculation with avirulent *P. parasitica* Hind 4 compared with extracts prepared from non-inoculated PALGFP (■) and inoculated non-transgenic plants (●). Bars represent standard error of the mean ($n = 3$). (c) Time course of the production of GFP in PALGFP following inoculation with the avirulent *P.'parasitica* Hind 4 (○) and the virulent *P. parasitica* Noks 1 (●). Note that there is no data for the final time point for the virulent interaction because the leaf tissue was completely degraded. Bars represent the standard error of the mean ($n = 3$).

A time course of the production of GFP in the virulent and avirulent interactions with *P. parasitica* was conducted (Figure 6c). Maximal expression of GFP in the avirulent interaction with isolate Hind 4 was found 120 h post inoculation. For the virulent interaction with isolate Noks 1 there was only low level expression of GFP with a maximum at 168 h post inoculation. No data was collected for the virulent interaction at 264 h after inoculation because the leaf tissue had completely degraded.

Discussion

We have shown that the GFP can be used as a spatial and temporal reporter of defence gene induction. The *PAL1* promoter–GFP reporter system used allowed non-invasive visualisation of PAL gene activity using conventional epifluorescence microscopy of living cells that had been subjected to wounding, heavy metal exposure or inoculation by two types of pathogen. The sensitivity of this system is very high and GFP production can be shown at the single cell level. These findings complement previous work that used GUS as a reporter to describe PAL gene expression patterns in *A. thaliana* (Mauch-Mani and Slusarenko, 1996; Ohl et al., 1990; Wanner et al., 1995).

Using the GFP reporter we have shown that when plants were grown and inoculated under identical conditions to those of the previous studies there was an earlier induction of PAL expression within cells and tissues than has previously been reported. For example, using GUS as the reporter, Mauch-Mani and Slusarenko (1996) found *PAL1* to be activated after 120 h in the incompatible interaction of *P. parasitica* with *A. thaliana* and did not show any activation in compatible interactions. We have found *PAL1* activation in the incompatible interaction within 72 h after inoculation and low level activation in the compatible interaction after 120 h. A limitation of both GUS and GFP reporter systems is that transient gene expression cannot be readily visualised. The half life of GFP, for example has been shown to be greater than 24 h and fluorescence may be maintained for several days (Casper and Holt, 1996; Sheen et al., 1995). Following

pathogen attack PAL mRNA levels in *A. thaliana* follow a transient expression pattern (Dong et al., 1991) that cannot be shown using GFP because of the stability of the protein once formed. The declining levels of GFP revealed via both microscopy and spectrofluorometry cannot therefore, be attributed directly to a decline in activity of *PAL1* gene expression.

Spectrofluorometry showed that the relative amounts of GFP produced following pathogen attack can be quantified. In particular, clear quantitative differences in GFP levels occurred in the incompatible combination of *P. parasitica* (Hind 4) with *A. thaliana* compared with the compatible interaction with *P. parasitica* (Noks 1). One consideration for the use of GFP in plant pathogen interactions is the accumulation of autofluorescent compounds in challenged cells and a subsequent increase in overall fluorescence of the tissue. Autofluorescence can, however, be accounted for by the use of correct controls.

The *PAL1* promoter–GFP reporter system appears to be as sensitive as the GUS system when used under similar conditions, even though the latter uses enzymatic amplification, although we have not tested this using our transgenic plants. Under some conditions GFP appears to be more sensitive. For example, in a study that used a *PAL1*-GUS enzyme activity assay GUS activity in *A. thaliana* leaf cells was detected after 8 h exposure to $HgCl_2$ (Ohl et al., 1990) whereas *PAL1*-GFP activation was visualised directly within 2–3 h of $HgCl_2$ exposure. Measurement of GUS activity requires destruction of cells but using luciferase as a reporter (*lux F* gene) Giacomin and Szalay (1996) showed *PAL1* promoter activity within live cells. While this approach enabled real time analysis of gene activity, the bioluminescence produced by luciferase required the use of photon imaging. Photon imaging does not provide the resolution and clarity that can be achieved with GFP using fluorescence microscopy. The use of GFP as a reporter gene also enables visualisation of more subtle changes in gene expression than *lux F*. For example, in the incompatible interaction between *A. thaliana* and *Ps. syringae* pv. *tomato* GFP expression was detected within 12–18 h of inoculation compared with 24 h for *lux F*.

Our results confirm the differences in major defence gene activity in compatible and incompatible interactions of *A. thaliana* with both *P. parasitica* and *Ps. syringae* pv. *tomato*. In addition, we have demonstrated the spatial induction of PAL within individual lesions. Our time course study showed that cells that are initially challenged by a pathogen respond quickly by the production of PAL. Cells that are adjacent to penetrated cells also respond by producing PAL even though they may not be in direct contact with the pathogen. This suggests that a signal for PAL gene transcription had been transmitted to cells adjacent to penetration sites. The 'halo' of bright fluorescence that extends for several cell layers around established incompatible lesions shows high levels of PAL production, indicating a close association of the activation of the phenylpropanoid pathway with lesion containment.

During pathogen attack, defence-related activation of PAL genes in *A. thaliana* has been demonstrated in incompatible interactions with both the oomycete and bacterial pathogens used in the present study and also with *Xanthomonas campestris* (Giacomin and Szalay, 1996; Lummerzheim et al., 1993; Ohl et al., 1990; Wanner et al., 1995). Although PAL genes are up-regulated during these interactions, the exact role of phenylpropanoids in defence in *A. thaliana* is still unclear (Hagemeier et al., 2000). One phenylpropanoid derivative that is strongly implicated in defence in *A. thaliana* is salicylic acid. The concentration of salicylic acid increases within incompatible tissues and it is thought to be directly involved in cellular signalling during incompatible responses (Mauch-Mani and Slusarenko, 1996; Thomma et al., 2001). Other defence responses that involve the phenylpropanoid pathway include lignification of cell walls following pathogen attack. In interactions of *A. thaliana* with *X. campestris* pv. *campestris* and *Ps. syringae* pv. *maculicola* increased lignin biosynthesis has been correlated with incompatibility (Lauvergeat et al., 2001; Lee et al., 2001). In the present study GFP was found to be localised within cells that reacted incompatibly with both *P. parasitica* and *Ps. syringae* pv. *tomato* and in surrounding cell layers, suggesting that phenylpropanoid products such as lignin had accumulated. In recent experiments, lignin accumulation in cells and cell walls following inoculation with an avirulent isolate of *P. parasitica* was shown to follow the expression pattern of GFP (data not shown).

GFP expression could be readily visualised in several tissue and cell types of *A. thaliana* but it was especially useful for analysis of PAL activity in leaf cells. The fluorescence induced in GFP by exposure to ultraviolet or blue light meant that live cells could be directly analysed after a variety of treatments over a prolonged time course. As was observed by Sheen et al. (1995) the bright red autofluorescence of chlorophyll pigments under blue light excitation of leaf cells tended to decrease the relative intensity of GFP. The reduced

intensity was overcome by the use of ultraviolet light that produced a vivid green fluorescence against an essentially blue background. One benefit of using blue light rather than ultraviolet light was that changes in cellular chemistry associated with cell death could be visualised by the bright yellow autofluorescence induced in affected cells and cell walls.

In conclusion, the spatial and temporal induction of *PAL1* gene expression during interactions with virulent and avirulent isolates of *Ps. syringae* pv. *tomato* and *P. parasitica* has been analysed using a GFP reporter system. The GFP system has several advantages over other reporter systems especially in the ease of sample preparation and the ability to observe the dynamics of interactions in live cells.

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