Journal of Experimental Botany, Vol. 56, No. 421, pp. 2983–2994, November 2005
doi:10.1093/jxb/eri295 Advance Access publication 5 October, 2005
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Journal of Experimental Botany

RESEARCH PAPER

Stress responses to polycyclic aromatic hydrocarbons in *Arabidopsis* include growth inhibition and hypersensitive response-like symptoms

Merianne Alkio[1], Tomoko M. Tabuchi[1], Xuchen Wang[2] and Adán Colón-Carmona[1,*]

[1] *Department of Biology and Department of Environmental, University of Massachusetts Boston, 100 Morrissey Blvd., Boston, MA 02125, USA*

[2] *Earth and Ocean Sciences, University of Massachusetts Boston, 100 Morrissey Blvd., Boston, MA 02125, USA*

Received 30 March 2005; Accepted 22 August 2005

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are of global environmental concern because they cause many health problems including cancer and inflammation of tissue in humans. Plants are important in removing PAHs from the atmosphere; yet, information on the physiology, cell and molecular biology, and biochemistry of PAH stress responses in plants is lacking. The PAH stress response was studied in *Arabidopsis* (*Arabidopsis thaliana*) exposed to the three-ring aromatic compound, phenanthrene. Morphological symptoms of PAH stress were growth reduction of the root and shoot, deformed trichomes, reduced root hairs, chlorosis, late flowering, and the appearance of white spots, which later developed into necrotic lesions. At the tissue and cellular levels, plants experienced oxidative stress. This was indicated by localized H_2O_2 production and cell death, which were detected using 3, 3′-diaminobenzidine and trypan blue staining, respectively. Gas chromatography-mass spectrometry and fluorescence spectrometry analyses showed that phenanthrene is internalized by the plant. Gene expression of the cell wall-loosening protein expansin was repressed, whereas gene expression of the pathogenesis related protein PR1 was induced in response to PAH exposure. These findings show that (i) *Arabidopsis* takes up phenanthrene, suggesting possible degradation in plants, (ii) a PAH response in plants and animals may share similar stress mechanisms, since in animal cells detoxification of PAHs also results in oxidative stress, and (iii) plant specific defence mechanisms contribute to PAH stress response in *Arabidopsis*.

Key words: Cell death, oxidative stress, phytoremediation, polycyclic aromatic hydrocarbons (PAHs), trichomes.

Introduction

With increasing environmental pollution and anthropological disturbances to ecosystems, the study of abiotic stress responses in plants has become ever more important in agriculture, forest management, and ecosystem restoration strategies. Ozone, for instance, has been shown to compromise forest and crop species leading to reduced yields (Preston and Tingey, 1988; Heagle, 1989; Pell *et al.*, 1997), whereas organic pollutants accumulate in vegetation (Simonich and Hites, 1994; reviewed in Pilot-Smits, 2005) and can cause health problems (reviewed in Harvey, 1991; Pitot and Dragan, 1996). Some of the best studied abiotic stress inducers include heavy metals, ozone, UV light, salinity, and drought. Experimentation on the toxicology and physiology of abiotic stress inducers has resulted in significant advances in environmental protection and agriculture.

For example, studies on metal toxicity in plants have led to the identification of transport proteins. This has advanced bioremediation of metal-contaminated soil environments, where some plants have been shown to internalize toxic elements such as cadmium (Salt *et al.*, 1995), arsenic (Ma *et al.*, 2001), and nickel (Krämer *et al.*, 2000). The molecular knowledge of uptake, transformation, and storage of toxic metals and their derivatives in plants have led to promising biotechnological applications (Bizily *et al.*, 2000; Mejáre and Bülow, 2001; Dhankher *et al.*, 2002; Song *et al.*, 2003). In addition, studies of ozone-induced stress have shown that oxidative stress leads to cell death

* To whom correspondence should be addressed. Fax: +1 617 287 6650. E-mail: adan.colon-carmona@umb.edu

(reviewed in Rao and Davis, 2001). The necrosis of tissues involves salicylic acid (SA), jasmonic acid (JA), and ethylene signalling processes (Rao *et al.*, 2000, 2002; Vahala *et al.*, 2003). Moreover, common signalling molecules have been identified for abiotic and biotic stress pathways. For instance, mitogen-activated protein kinases (MAPKs) mediate responses to both ozone and pathogen exposure (Sharma *et al.*, 1996; Samuel *et al.*, 2000; Kovtun *et al.*, 2000; reviewed in Rao and Davis, 2001; Vranova *et al.*, 2002).

The hazardous pollutants polycyclic aromatic hydrocarbons (PAHs) are poorly studied stress inducers. PAHs consist of two or more benzene rings, which make these molecules highly persistent in the environment. Examples include naphthalene, phenanthrene, benz[*a*]anthracene, and benz[*a*]pyrene, which contain 2–5 aromatic rings, respectively. Many PAHs in the environment are by-products in petroleum-based manufacturing; they are present in coal tar, asphalt, tyres, and other oil-containing products and are released into the environment during incomplete combustion, and through oil spills, for instance (Committee on *In Situ* Bioremediation, 1993). In animals, PAHs have a broad impact on health. They interact with DNA resulting in mutations and cancer, benz[*a*]pyrene being one of the most toxic and carcinogenic compounds known (Harvey, 1991; Anonymous, 2002). In animal cells PAHs are transformed by oxidases and reductases (Burczynski *et al.*, 1999; Burchiel and Luster, 2001). PAH metabolism generates reactive electrophilic metabolites, which are the actual carcinogenic compounds that cause DNA damage (Wang *et al.*, 2002). Furthermore, PAHs trigger the production of reactive oxygen species and cell death in animal cells (Flowers *et al.*, 1997; Burcyznski *et al.*, 1999; Hiura *et al.*, 1999).

Various trees (such as poplar and jack pine; Rentz *et al.*, 2003), grasses (rye, oat, wheat, and maize; Aprill and Sims, 1990) and other agricultural crops (sunflower, soybean, pea, canola, and carrot; Wild and Jones, 1992; Liste and Alexander, 2000) have been shown to tolerate crude oil pollutants in soil (Harvey *et al.*, 2002). As in the hyperaccumulation and detoxification of heavy metals, most elaborate approaches for organic pollutant removal include genetic engineering of plants specifically to target the contaminant. Promising results have been obtained with plants expressing bacterial enzymes to detoxify trinitrotoluene (Hannink *et al.*, 2001), or overexpressing a plant enzyme to degrade trichlorphenol and other phenolic compounds (Wang *et al.*, 2004). Such innovative work in plants has become possible only after decades of research on bacterial degradation of organics, which has led to identifying strains capable of using aromatic compounds as carbon sources, isolating their biodegrading genes, and engineering bacterial species with the appropriate genes for their introduction into contaminated sites (Sisler and Zobell, 1947; Treccani *et al.*, 1954; reviewed in Pieper and Reineke, 2000).

In contrast to micro-organisms and animals, little is known about the cellular and molecular mechanisms of how plants take up, respond to, and degrade PAHs (Cobbett and Meagher, 2002; Harvey *et al.*, 2002). Generally, xenobiotic detoxification in plants involves transformation, conjugation for example with glucose or glutathione, and sequestration in the vacuole or cell wall (McCutcheon and Schnoor, 2003). Some PAHs like benz[*a*]pyrene (Sandermann *et al.*, 1984) and fluoranthene (Kolb and Harms, 2000) have been shown to be metabolized in cell cultures of different plant species. Because of the high hydrophobicity of PAHs, they are generally assumed to be tightly bound to soil particles and hardly bioavailable (Pilon-Smits, 2005), but some studies indicate that plants do take up PAHs from soil (Samsoe-Petersen *et al.*, 2002; Kuhn *et al.*, 2004). However, the plant genes responsible for PAH uptake, degradation, and conjugation are largely unknown. Mechanisms of the PAH toxicity to plants are poorly understood, and the phytotoxicity appears to vary depending on the particular PAH and plant species (Wittig *et al.*, 2003; Baek *et al.*, 2004). In addition to degradation and detoxification, molecular knowledge about the stress response and defence mechanisms involved is required for finding ways to support plant tolerance towards PAHs. Presently, it is not clear to what extent PAH exposure triggers stress signalling pathways common to other abiotic or biotic stresses in plants, and whether signalling components specific to PAH stress exist.

The objective of this study was to investigate the physiological, cellular, and molecular details of PAH stress responses in *Arabidopsis* (*Arabidopsis thaliana*). Because many PAHs are reminiscent of plant hormones and secondary metabolites, plants treated with phenanthrene were expected to internalize the model PAH and exhibit altered growth, morphology, and gene expression. Investigations on the stress response were also expected to provide insights into plant PAH monitoring, degradation and stress signalling mechanisms.

Materials and methods

Plants and growth conditions

Seeds from *Arabidopsis thaliana* ecotype Columbia plants were used in most experiments. For tissue-specific localization of the glutathione-*S*-transferase expression, seeds from the *AtGSTF2* promoter::β-glucuronidase reporter plant line (*GSTF2::GUS*) were used (Smith *et al.*, 2003). The construct contains 1050 bp of the *GSTF2* (At4g02520) promoter fused to the β-glucuronidase (GUS) ORF in the binary vector pBI101 (Smith *et al.*, 2003). Seeds were surface-sterilized and stratified at 4 °C for 3 d. For control plants, which were grown without the presence of phenanthrene, square Petri dishes containing solid growth medium were prepared with half-strength Murashige and Skoog (MS) medium supplemented with 1.5% w/v sucrose and 0.9% w/v phytoagar, pH 5.7. Phenanthrene has an octanol:water distribution coefficient, log K_{ow}, of 4.45–4.57, reflecting its high hydrophobicity. With water solubility of 1–1.6 mg l^{-1} it is practically insoluble in water. Therefore, special care was taken in preparing growth medium with the PAH. Phenanthrene containing

Petri dishes were prepared by adding 7.5–175 µl of 100 mM phenanthrene in methanol to aliquots of 35 ml of MS medium to achieve concentrations of 0.05–0.75 mM phenanthrene. The aliquots were autoclaved, mixed well while hot, and poured into Petri dishes. Seeds were sown in the Petri dishes, which were then incubated vertically under long-day conditions (16/8 h photoperiod at approximately 130 µmol photons m^{-2} s^{-1} at 23±1 °C day and night) for 7–35 d as indicated in the text. Plants were observed under a Zeiss 2000-C dissection microscope equipped with an Olympus 340 digital camera.

Histochemistry and ion leakage

Trypan blue staining of dead cells was performed as described by Belenghi *et al.* (2003). Staining for the presence of H_2O_2 by the DAB uptake method was performed as described by Thordal-Christensen *et al.* (1997). GUS-staining was performed as described by Jefferson *et al.* (1987). For all histochemical methods whole plants, shoots or detached leaves were taken and photographed under a Zeiss 2000-C dissecting microscope equipped with an Olympus 340 digital camera before and after staining. Per treatment at least six plants were stained with each method. To quantify cell death, relative ion leakage was determined by a method modified from Vahala *et al.* (2003). Shoots of 26-d-old plants were rinsed twice in double distilled (dd) water and placed in 10 ml of dd water. After 20 h of incubation at room temperature ion leakage was measured with a conductivity meter (YSI Model 35, Yellow Springs Instrument Co. Inc., OH). Samples were autoclaved to kill tissue and conductivity was measured again to determine total ion content. Ion leakage was expressed as the percentage of total ion content. Per treatment at least three samples consisting of 3–10 plants each were measured.

RT-PCR

Gene expression was analysed in 21-d-old plants grown on 0 or 0.25 mM phenanthrene by applying the reverse transcriptase–polymerase chain reaction (RT–PCR). Total RNA from whole plants was isolated using the RNeasy Plant Mini Kit (Qiagen USA). First-strand cDNA was synthesized using SuperscriptII protocol (Invitrogen). Following primers were used in the PCR: elongation initiation factor 4A (*EIF4A*; At3g13920), 5′-CTCTCGCAATCTTCGCTCTTCTCTTT-3′ and 5′-TTCTCAAAAC-CATAAGCATAAATACCC-3′; actin 7 (*ACT7*; At5g09810), 5′-GGTCGTCCTAGGCACACTGGTG-3′ and 5′-ACAATACCGGTTGTACGACCAC-3′; expansin 8 (*EXP8*; At2g40610), 5′-ATGGGCGGAGCTTGTGGCTATG-3′ and 5′-ACCTCAGCAATATGCAGCCACG-3′; pathogenesis related protein 1 (*PR1*; At2g14610), 5′-GCAGACTCATACACTCTGGTGG-3′ and 5′-ACTCCATTGCACGTGTTCGCAG-3′; glutathione-*S*-transferase F2 (*GSTF2*; At4g02520), 5′-TCGACCCAGTGGCTTCAAAGC-3′ and 5′-TTGGGCAATGAGAAAGCC-3′. PCR reactions were carried out by *Taq* Polymerase (Invitrogen) with following program: an initial 2 min at 94 °C followed by 25 cycles of 1 min at 94 °C, 50 s at 58.0, 59.1, or 61.0 °C (annealing temperatures for *EIF4A*/*ACT7*, *PR1*, and *EXP8*/*GSTF2*, respectively), 1 min at 72 °C; and a final extension of 4 min at 72 °C. Gene expression was analysed in two independently grown, harvested, and extracted sets of samples (biological replicates) of 5–10 plants each and the results were similar.

Spectrofluorometric and gas chromatography-mass spectrometry (GC-MS) analyses

Extracts were prepared from 30 d old control and phenanthrene treated plants. Shoot and root tissues from 12 to 24 plants were separated for analysis. 0.125–0.541 g of tissue was quickly washed twice in hexane to remove residual phenanthrene, frozen in liquid nitrogen, ground to fine powder with a mortar and pestle, and extracted with dichloromethane:methanol (9:1, v/v). Extracts were passed through a Na_2SO_4 column to remove water. Extracts were excited at 337 nm, and then emission scanned using a PTI QM-1 spectrofluorometer (Photon Technology International Inc.). For the GC-MS, extracts were concentrated into approximately 100 µl and analysed with a Finnigan Voyager Gas Chromatography-Mass Spectrometer. The GC was equipped with a split/splitless injector and an AS800 auto-sampler. One µl of concentrated samples was injected into the column and aromatic compounds were separated using a DB-5MS 30×0.25 mm fused silica column (J&W Scientific) programmed from 50 °C to 300 °C at the rate of 6 °C min^{-1} and held at 300 °C for 10 min. Helium was used as a carrier gas and the flow rate was set at 1 ml min^{-1}. The mass spectrum was scanned from 50–600 nm in the Electron Ionization (EI) mode (70 eV). Phenanthrene was identified based on the retention time and ion *m*/*z* ratio of authentic PAH mixed standard (Sigma). Analyses were carried out for two independent biological replicates and the results were similar.

Fluorescence microscopy

The leaves from plants growing at 0, 0.25, or 0.5 mM phenanthrene were mounted on a slide glass with glycerol and viewed under an Olympus BX60 compound microscope with an U-MWU cube (excitation filter BA420), and photographed with an Olympus 340 digital camera.

Results

Arabidopsis *stress response to phenanthrene*

To characterize plant responses to a PAH, *Arabidopsis* was grown on phenanthrene containing medium under sterile conditions. Phenanthrene-treated plants exhibited many stress characteristics, such as reduction of root growth and reduction in size and number of leaves (Fig. 1). Root hair development was drastically inhibited (Fig. 1C–F). Trichomes were deformed and reduced in size and number (Fig. 2E, F). Between 0.05 mM and 0.25 mM phenanthrene, the severity of the stress symptoms increased with concentration. Plants grown on 0.25, 0.5, or 0.75 mM phenanthrene appeared equally stressed, indicating a saturation of the response. Concentrations higher than 1.0 mM were excessively toxic and killed the plants after 30 d of growth.

When *Arabidopsis* was grown on phenanthrene concentrations higher than 0.05 mM, plants exhibited white spots, which developed into necrotic lesions (Fig. 2B, D–H). Note in Fig. 2D the glossy, compact, slightly upward curved leaves, which were typical for plants grown on phenanthrene. The white spots were most prominent in leaves, starting in cotyledons (Fig. 2B), but were also present on the hypocotyl, flowers, and stems.

Necrotic lesions are a symptom of a hypersensitive response (HR) in an avirulent plant–pathogen interaction, in which plant defence mechanisms include local cell death to restrict the spread of the pathogen (Lamb and Dixon, 1997). Therefore, an attempt was made to find out if there were further parallels to an HR. Firstly, to determine whether the white spots and necrotic looking patches contained dead cells, trypan blue staining was used. Phenanthrene-exposed plants exhibited blue spots indicating dye accumulation in

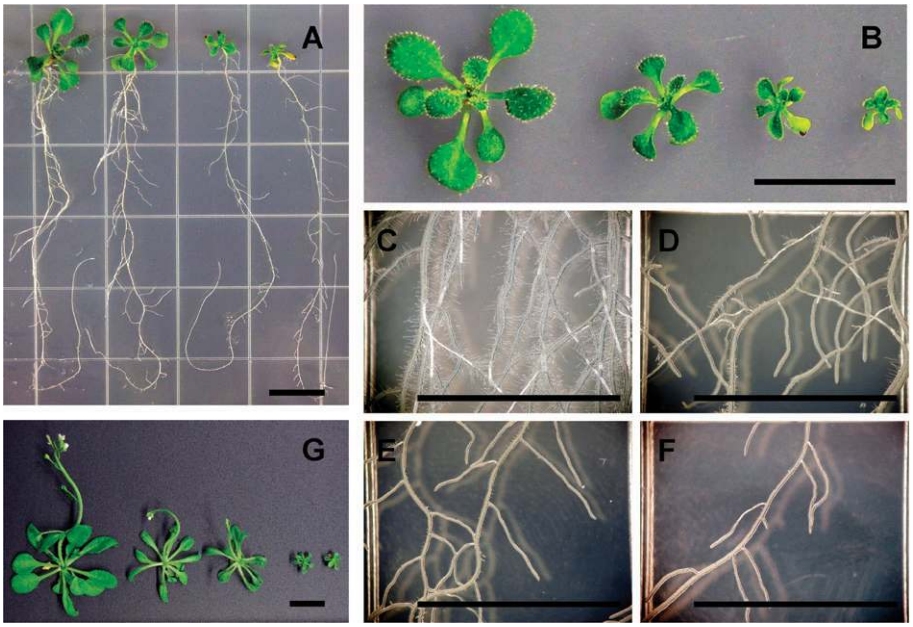

Fig. 1. Phenanthrene inhibits root and shoot growth. *Arabidopsis* was grown on medium supplemented with 0–0.5 mM phenanthrene. Square plates were positioned vertically in a plant growth incubator under long days at 23 °C. At each concentration, at least 24 plants were analysed. (A–F) 14-d-old plants. Representative plants (A) or shoots (B) grown on 0, 0.05, 0.1, or 0.25 mM phenanthrene (left to right) were transferred onto the same underlay before imaging. Roots at the same relative position of the root system at 0 (C), 0.05 (D), 0.1 (E), and 0.25 (F) mM phenanthrene. (G) Shoots of 28-d-old plants grown on 0, 0.05, 0.1, 0.25, or 0.5 mM phenanthrene (from left to right). Scale bars 10 mm.

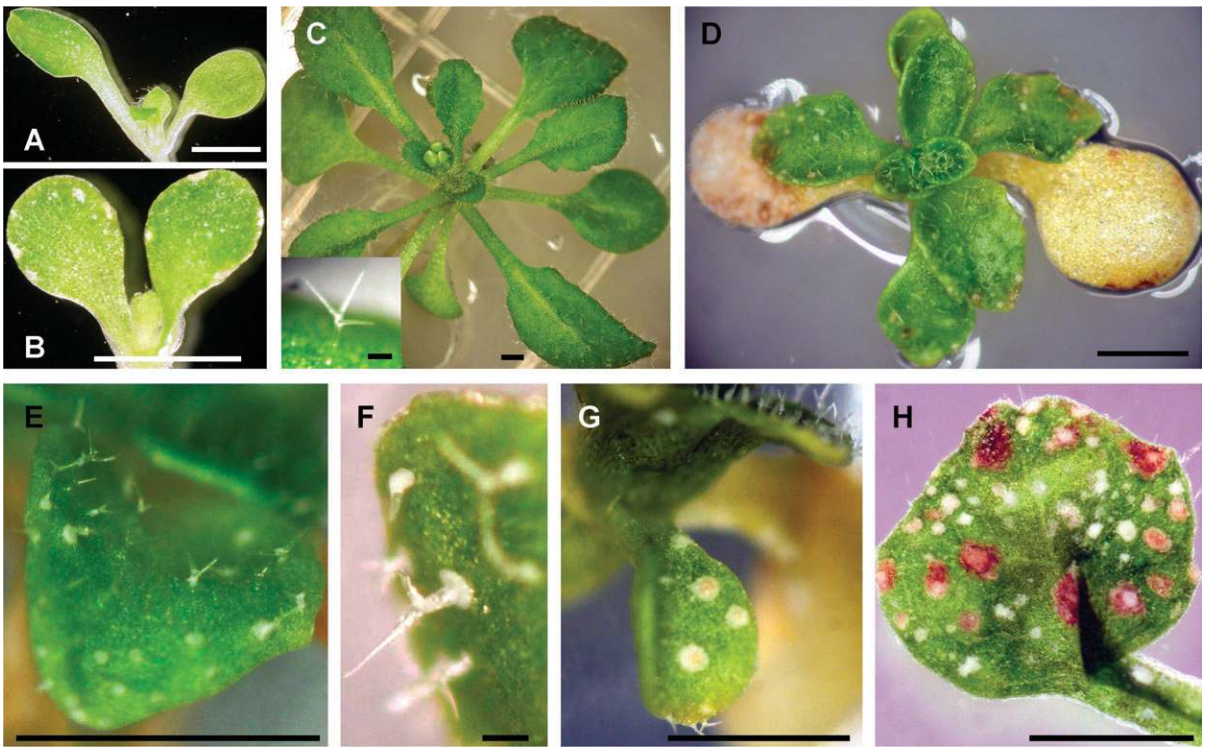

Fig. 2. Phenanthrene causes white spots and necrotic lesions on leaves. *Arabidopsis* was grown on medium supplemented with 0–0.5 mM phenanthrene under long days at 23 °C. (A, B) 7-d-old seedlings grown on 0 (A) or 0.5 mM (B) phenanthrene. (C, D) 21-d-old plants grown on 0 (C) or 0.25 mM (D) phenanthrene. Inset in (C) shows a trichome on a plant grown on 0 mM phenanthrene. (E–H) Leaves of plants grown on 0.5 mM phenanthrene for 21 (G), 31 (E, F), or 34 d (H). Scale bars 1 mm, except 100 μm in (C, inset) and in (F).

dead cells (Fig. 3D, E). The frequency and size of the cell death events and lesions on leaves were dependent on the phenanthrene concentration in the medium, and the duration of exposure (data not shown). When plants were initially grown without phenanthrene for 8 d, and then transferred to medium containing 0.25, 0.5, or 0.75 mM phenanthrene, dead cells were detected as early as 6 h after the start of exposure. After 12 h of exposure, 50% of the plants exhibited cell death. At this time point, white spots became visible under the dissection microscope. Figure 3C, D show a leaf 48 h after transfer to 0.5 mM phenanthrene. Blue spots indicating dead cells in Fig. 3D co-localize with white spots in Fig. 3C, which shows the same leaf before staining. In addition, conductance measurements indicated increased ion leakage at higher phenanthrene concentrations (Fig. 3F), strengthening the hypothesis that plants were experiencing cell death as a result of phenanthrene exposure.

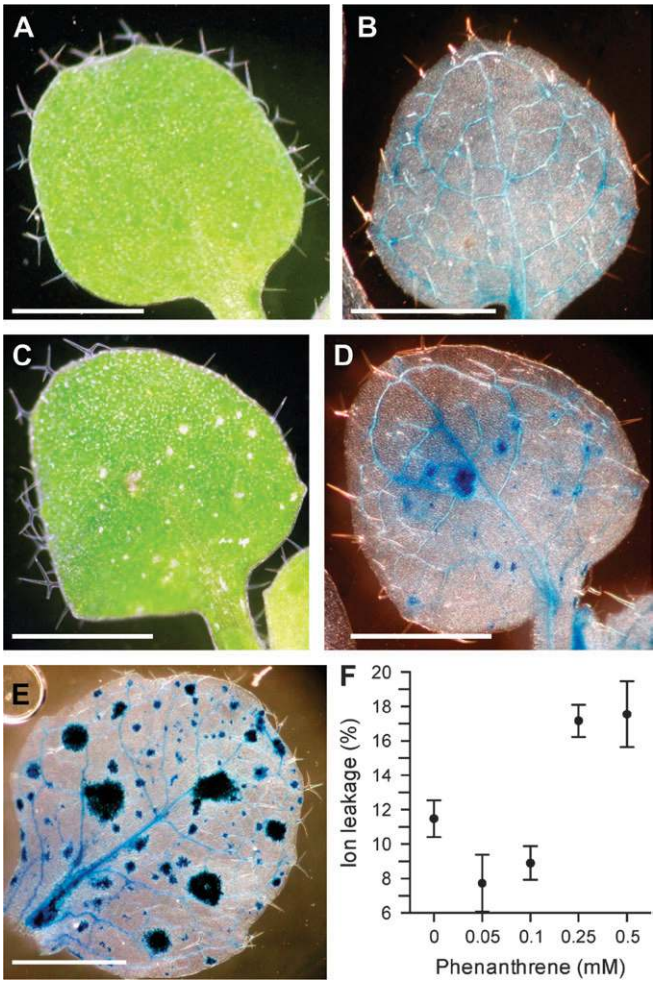

Fig. 3. Phenanthrene exposure promotes cell death. (A–D) *Arabidopsis* was grown on medium without phenanthrene under long days at 23 °C for 8 d before transfer to 0 (A, B) or 0.5 mM (C, D) phenanthrene for 48 h. Leaves were photographed before (A, C) and after (B, D) staining with trypan blue. (E) Leaf of a plant grown continuously on 0.5 mM phenanthrene for 60 d before staining with trypan blue. Dark blue spots in (D) and (E) indicate dye accumulation in dead cells. (F) Ion leakage (as the percentage of total ion content) into distilled water during 20 h of incubation at room temperature from in 26-d-old plants grown on 0–0.5 mM phenanthrene. Data points show mean ±standard deviation; n=4 for 0, 0.05, and 0.1 mM phenanthrene, n=3 for 0.25 and 0.5 mM phenanthrene. Scale bars in (A–E) 1 mm.

In an HR, reactive oxygen species (ROS) such as hydrogen peroxide are known to mediate cell death in exposed tissues (Lamb and Dixon, 1997). Therefore H_2O_2 production was analysed in phenanthrene-treated plants using the 3,3′-diaminobenzidine (DAB) uptake method. Polymerization of DAB, visible as a brown precipitate in the presence of H_2O_2, was detected in leaves (Fig. 4C, H, J, K). Notably, like cell death, H_2O_2 production co-localized with white and necrotic spots, where present (Fig. 4I, J). Figure 5 summarizes the time-course of H_2O_2 production and cell death after transferring 8-d-old plants from control medium (0 mM phenanthrene) to 0.25, 0.5, or 0.75 mM phenanthrene-containing environments. Because the frequency of the symptoms did not differ between the phenanthrene concentrations 0.25, 0.5, and 0.75 mM, the data from those plants were pooled at each time point (Fig. 5B). Phenanthrene-exposed plants started developing symptoms after 3–6 h of exposure, and the number of plants exhibiting symptoms increased with time. After 48 h of phenanthrene exposure 85% of the analysed plants exhibited white spots, dead cells, and H_2O_2 production (Fig. 5B). No white spots were found in the plants that were transferred to 0 mM phenanthrene (Fig. 5A). Positive DAB reaction in some plants at 3, 18, and 24 h after transfer to the 0 mM phenanthrene can be attributed to plant damage during transfer, because wounding also induces H_2O_2 production (Orozco-Cardenas and Ryan, 1999). After 48 h, no H_2O_2 production was detected in plants not treated with phenanthrene, indicating that plants had completely recovered from the transfer.

To reveal phenanthrene-induced changes in gene expression, reverse-transcription (RT) PCR experiments were carried out for selected genes in 21-d-old plants. The expression levels of the translation elongation initiation factor (*EIF4A*) and actin (*ACT7)* genes, which were used as a control for overall gene expression levels, were similar in plants grown on 0 mM and 0.25 mM phenanthrene (Fig. 6). Gene expression of expansin (*EXP8*) was examined because expansins have known roles in cell wall loosening and cell enlargement (Keller and Cosgrove, 1995; O'Malley and Lynn, 2000). In phenanthrene-treated plants the transcript levels of *EXP8* were reduced (Fig. 6). To identify a signalling pathway possibly involved in the phenanthrene response, gene expression levels were analysed of the pathogenesis related protein 1 (*PR1*), a SA-dependent marker gene in defence pathways and the HR (Alvarez *et al.*, 1998; Klessig *et al.*, 2000), and the glutathione-*S*-transferase (GST) gene *GSTF2*, which is known to be induced by ethylene, auxin, SA, paraquat, and sulphydryl compounds (Smith *et al.*, 2003). Phenanthrene-exposed plants showed an increase in

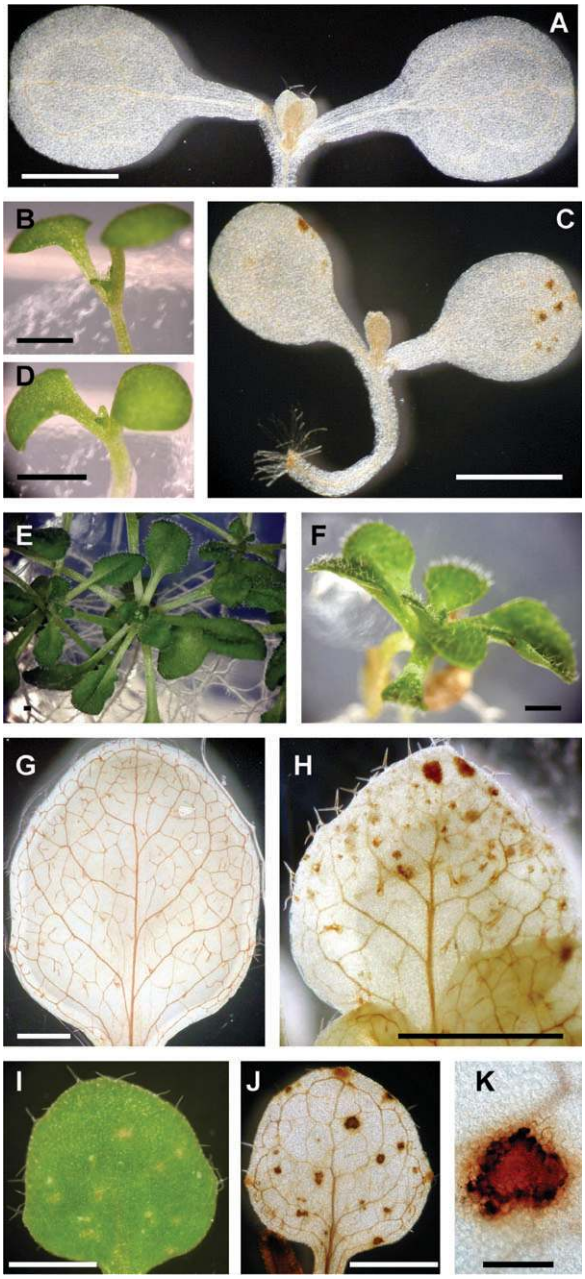

Fig. 4. Phenanthrene exposure promotes H_2O_2 production. *Arabidopsis* was grown on medium supplemented with 0–0.75 mM phenanthrene under long days at 23 °C. H_2O_2 was visualized by the DAB uptake method (A, C, G, H, J, K). Brown spots show polymerization of DAB in the presence of H_2O_2. Representative plants (B, D, E, F) or the leaf to be stained (I) were photographed prior to DAB staining. (A–D) 7-d-old seedlings grown on 0 (A, B) or 0.5 mM (C, D) phenanthrene. (E–H) 17-d-old plants grown on 0 (E, G) or 0.5 mM (F, H) phenanthrene. (I–K) A leaf from a plant transferred to 0.75 mM phenanthrene for 48 h after 8 d of growth on 0 mM phenanthrene. The leaf was photographed before (I) and after the staining (J). (K) Detail from (J) at higher magnification. Scale bars 1 mm except 100 μm in (K).

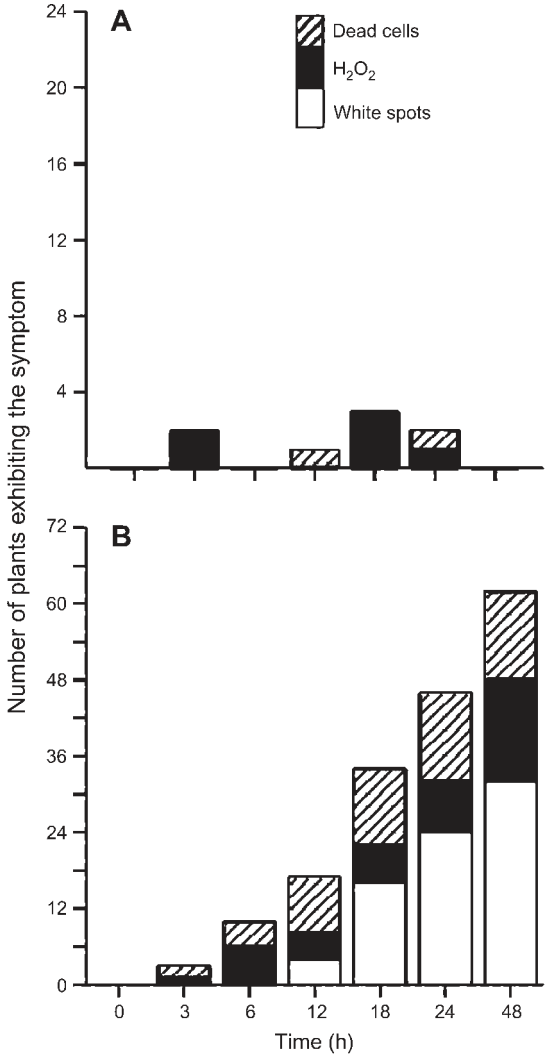

Fig. 5. The number of plants showings lesions (white bars), H_2O_2 production (black bars), and cell death (hatched bars) in leaves increases with the time of phenanthrene exposure. (A) Control plants, grown in the absence of phenanthrene; (B) plants treated with phenanthrene. A total of 336 plants were grown on medium without phenanthrene under long days at 23 °C for 8 d. Then 84 and 252 plants were transferred to media without and with phenanthrene, respectively. At 0, 3, 6, 12, 18, 24, and 48 h after transfer, 12 and 36 plants, on media without and with phenanthrene, respectively, were visually inspected for the presence of white spots using a dissection microscope. Afterwards, half of the plants from each treatment was stained with trypan blue to detect dead cells and the other half was stained with DAB to detect H_2O_2 production. Because at each time point each plant was examined for the presence of white spots and for dead cells or for H_2O_2 production, the number of observations is n=24 (A, control) and n=72 (B, phenanthrene exposure). The number of plants exhibiting stress symptoms when exposed to phenanthrene (B) increased monotonically over the seven time points. The probability that such an increase occurred by chance is (permutation test) 1/5040=0.0002.

mRNA steady-state levels for *PR1*, but not for *GSTF2* (Fig. 6). However, tissue-specific expression analysis using the *GSTF2::GUS* reporter line (Smith *et al.*, 2003) showed localized induction of *GSTF2* in leaves of 14-d-old phenanthrene-treated plants (Fig. 7B). These spatial expression patterns in leaves of *GSTF2::GUS* are reminiscent of H_2O_2 and trypan blue staining patterns observed in phenanthrene-exposed plants (Figs 3, 4).

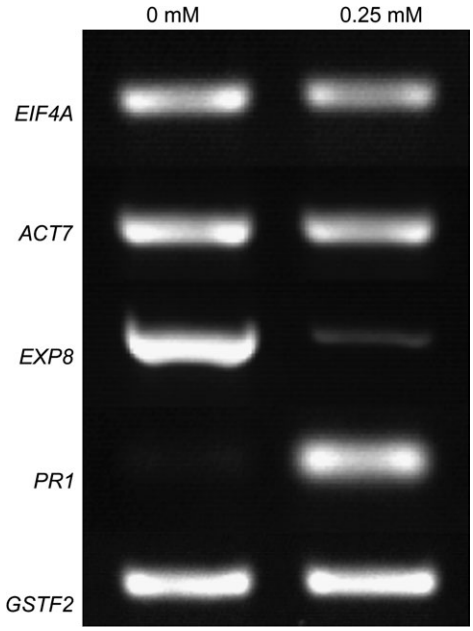

Fig. 6. Phenanthrene exposure affects gene expression. Total RNA was isolated from 21-d-old plants grown on 0 or 0.25 mM phenanthrene and used for RT-PCR experiments. Representative images of ethidium bromide-stained agarose gels showing relative mRNA levels for *EIF4A*, *ACT7*, *EXP8*, *PR1*, and *GSTF2* are presented. Relative expression levels of *EIF4A*, *ACT7*, *EXP8*, *PR1*, and *GSTF2* on 0.25 mM phenanthrene as compared to 0 mM phenanthrene were 0.95±0.046, 0.93±0.073, 0.27±0.111, 4.81±2.853, and 1.02±0.049, respectively (mean ±standard error of mean calculated using image analysis software Image J; n=2). For each PCR reaction with the gene specific primers 2 μl of the cDNA were taken, and 6 μLl of the PCR reaction was loaded on the gel.

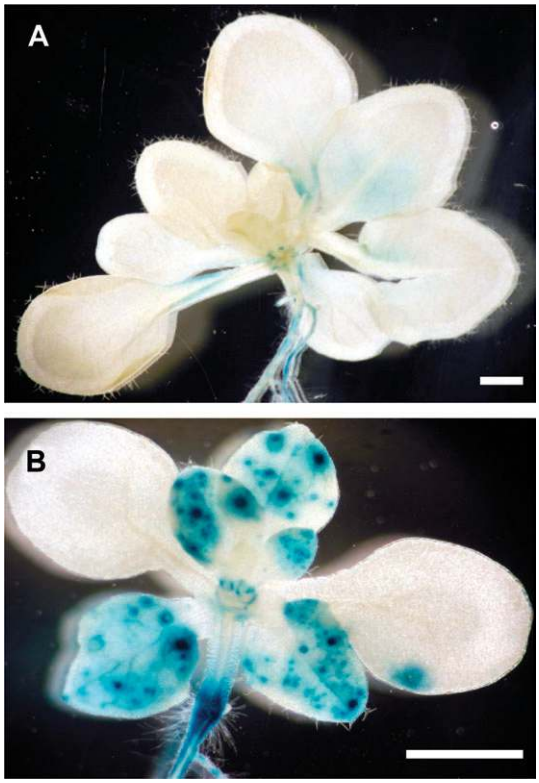

Fig. 7. Phenanthrene exposure induces localized *GSTF2::GUS* expression in leaves. *GSTF2::GUS* transgenic *Arabidopsis* plants were grown for 14 d on medium supplemented with 0 (A) or 0.25 (B) mM phenanthrene under long days at 23 °C. Plants were stained for 18 h in GUS staining buffer containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Scale bars 1 mm.

Phenanthrene is internalized by Arabidopsis

To verify that the observed responses to phenanthrene were due to its internalization, PAH uptake was analysed by gas chromatography and mass spectrometry (GC-MS), and fluorescence spectroscopy.

The total ion chromatograms presented in Fig. 8A and B provide evidence for phenanthrene uptake in *Arabidopsis*. The chromatograms of extracts from control (A) and phenanthrene-treated plants (B) show different patterns reflecting changes in the levels of many chemical compounds. In extracts from plants grown on 0.5 mM phenanthrene, the elution peak at 26.39 min, with a mass to charge (m/z) ratio of 178, (Fig. 8B), was identified as phenanthrene. No such peak was detected in extracts of control plants (Fig. 8A). Both root and leaf extracts contained phenanthrene (root data not shown).

Because phenanthrene, like other PAHs, is fluorescent when exposed to UV light and has a specific spectral emission signature, it can be detected using fluorescence spectroscopy (Dabestani and Ivanov, 1999). Phenanthrene in hexane has emission peaks at 347, 356, 364, and 374 nm after an excitation at 337 nm. Analysing fluorescence spectra of plant extracts, supporting evidence was obtained that phenanthrene is internalized by *Arabidopsis* (Fig. 8C, D). Both root and leaf extracts from phenanthrene-exposed plants exhibited emission spectra identical to the phenanthrene standard (root data not shown).

To determine if phenanthrene could be detected in plant tissue *in vivo*, plants grown in the presence or absence of phenanthrene were analysed using a fluorescence microscope. In 3-d-old seedlings no special fluorescence was detected, not even at the high concentration of 0.5 mM phenanthrene. From 7 d on, a clear difference in the fluorescence between control and phenanthrene-treated samples was observed (Fig. 9). In cotyledons of 7-d-old control plants, no notable fluorescence beyond the red autofluorescence of chlorophyll was seen (Fig. 9B). Some distinct, bright fluorescent spots were seen in the cotyledons of plants grown on 0.05 mM phenanthrene (not shown), and many more fluorescent spots were observed in the 0.5 mM phenanthrene treatment (Fig. 9D). In the 14- and 21-d-old plants grown on phenanthrene, fluorescence in true leaves was predominantly connected with trichomes (Fig. 9F, H, J, L, N, P). Particularly in young leaves, the fluorescence was always located in trichomes. Larger fluorescent patches developed with time, and were often centred on a collapsing trichome (Fig. 9L, N, P). Notably, the specific fluorescence, together with H_2O_2 production

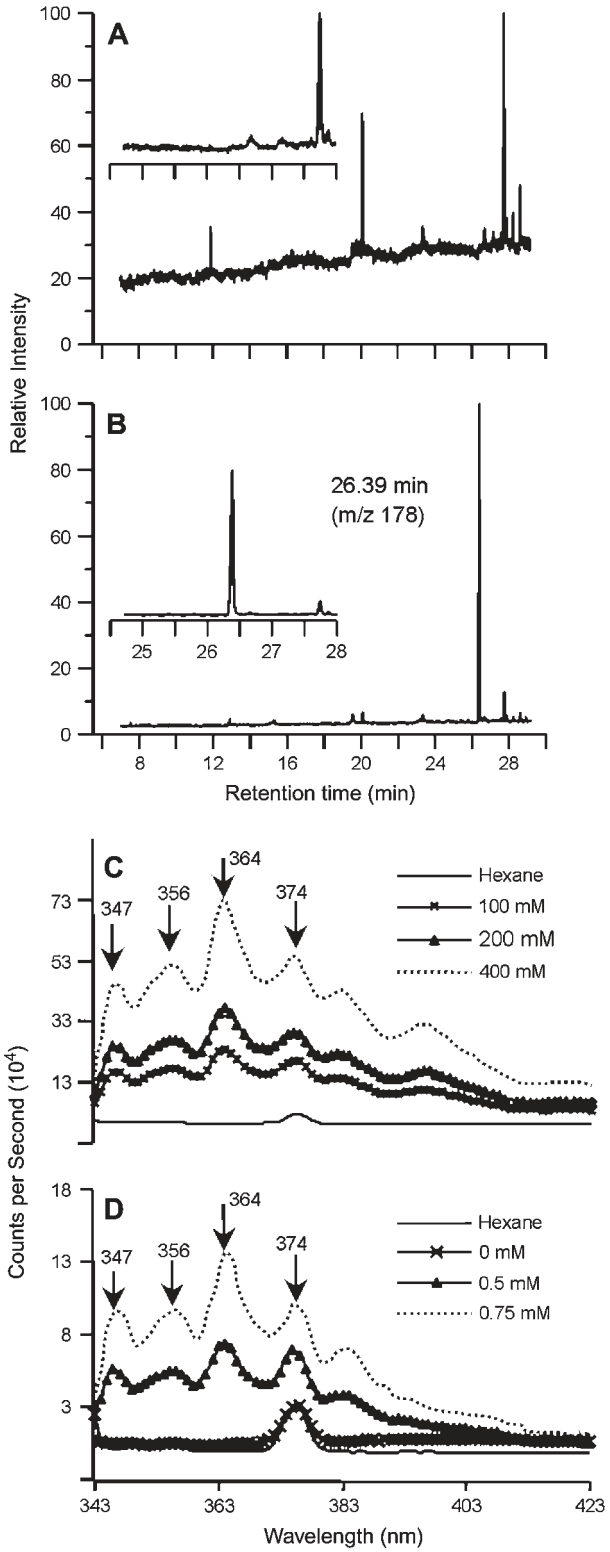

Fig. 8. Phenanthrene is found in root and leaf tissue. (A, B) Total ion chromatograms obtained with gas chromatography-mass spectrometry (GC-MS) analysis of leaf extracts from plants grown on 0 (A) or 0.5 mM (B) phenanthrene. Leaves were washed in hexane before freezing in liquid nitrogen. Ground tissue samples were extracted in dichloromethane:methanol (9:1, v/v) and analysed by GC-MS. The peak with the retention time of 26.39 min in (B) represents phenanthrene. Insets in (A) and (B) show the chromatograms for the retention time from 24.7 min to 28.0 min in detail. (C, D) Fluorescence spectra for phenanthrene standard solutions in hexane (C) and hexane extracts of leaves from plants grown at 0, 0.5, or 0.75 mM phenanthrene (D). Arrows indicate spectral peaks unique for phenanthrene.

and cell death, was also observed in phenanthrene-treated leaves, which did not yet contain visible necrotic lesions (i.e. plants treated with a low concentration of phenanthrene or for a short period of time), indicating that these events precede lesion formation.

Discussion

Despite the recognized importance of abiotic stresses in plants, little is known about the PAH stress responses and the mediating cellular signals. This report extensively documents the toxic nature of PAHs to plant growth. In addition, the experiments presented provide a foundation for PAH phytoremediation research in a genetically and molecularly tractable system such as *Arabidopsis*. In phytoremediation, plants, together with their associated microorganisms, are employed for decontamination (Cunningham and Ow, 1996). Phytoremediation has more public approval, and is far more cost-efficient in soil remediation compared with soil removal and incineration or disposal into landfills, or chemical extraction from removed soil (Pilon-Smits, 2005), adding an economic interest to studying stress responses to organic pollutants.

One prerequisite for using *Arabidopsis* as a model plant for PAH phytoremediation is that it takes up the compound under study. The GC-MS and fluorescence spectrometric analyses clearly show this to happen (Fig. 8). Present fluorescence microscopic analyses suggest that phenanthrene, or some phenanthrene derivative(s) accumulates in white spots seen under the dissection microscope (compare Figs 2, 9). Furthermore, the fluorescent spots (Fig. 9D, F, L, N, P) are reminiscent of the cell death patterns detected by trypan blue staining (Fig. 3D, E), suggesting that the accumulation locally causes cell death. While similar fluorescence observations have not been reported for plants growing on other abiotic stress inducers, it cannot be excluded that the fluorescence is due to endogenous fluorescent molecules that may be produced in response to PAH exposure as in wounding or in the HR. However, in the HR, fluorescent phenolic compounds are deposited in layers at or in the cell wall (Mellersh and Heath, 2001), while the fluorescence in the phenanthrene-treated plants is mostly in patches, seemingly inside the cytoplasm (e.g. Fig. 9J). The uptake of organic xenobiotics into the plant cell is often mediated by an ABC transport protein (Cobbett and Meagher, 2002), although simple diffusion is also thought possible (Pilon-Smits, 2005). Based on the hydrophobicity of the molecule, phenanthrene may passively penetrate the plasmalemma of root and shoot cells without

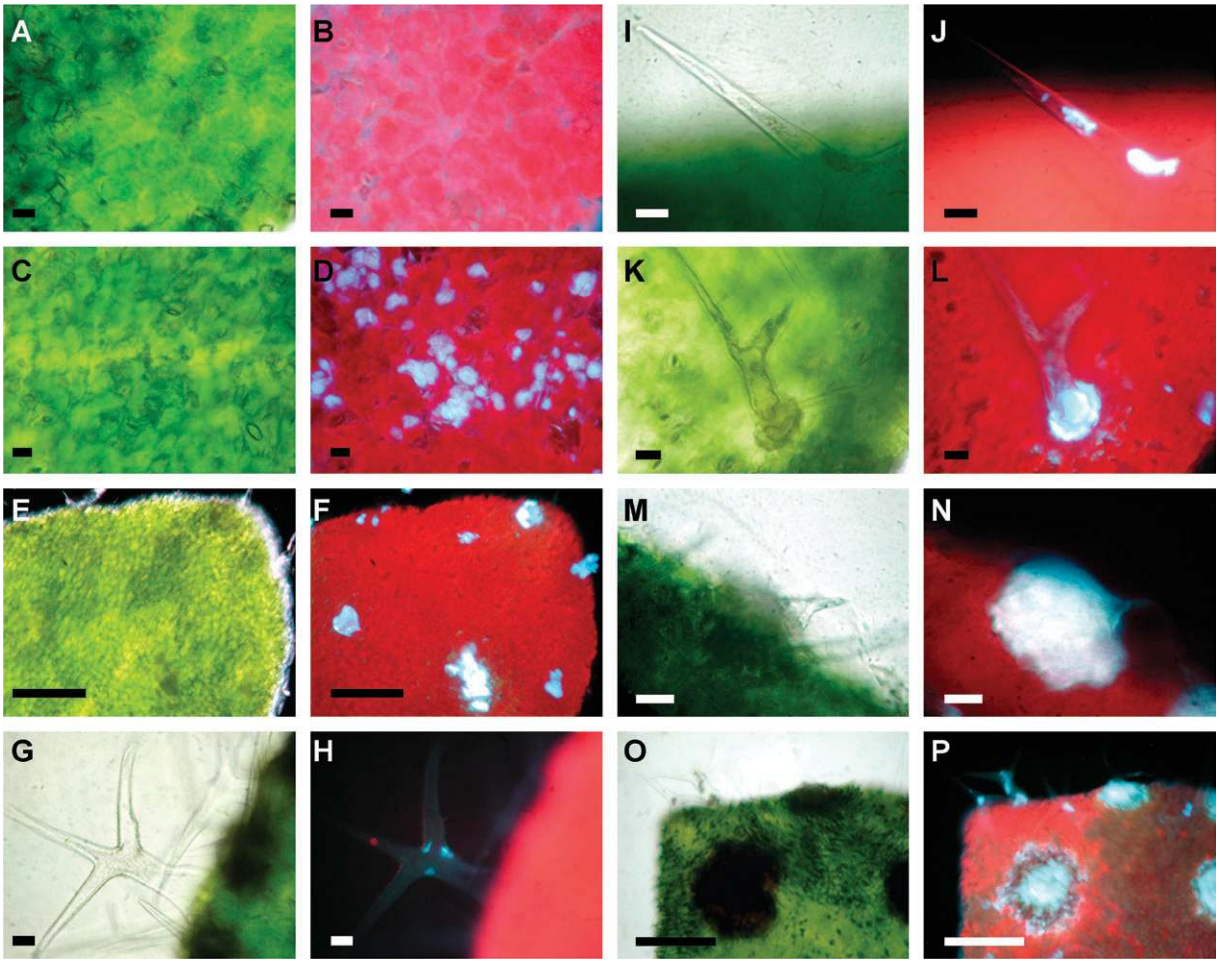

Fig. 9. Phenanthrene-grown plants exhibit specific fluorescence. *Arabidopsis* was grown for 7–28 d on medium supplemented with 0, 0.25, or 0.5 mM phenanthrene and observed at weekly intervals. Leaves were viewed with a compound microscope under bright field or UV illumination and images were captured with a digital camera. Two consecutive images (A, B, C, D etc) show the same site under bright field and UV illumination, respectively. (A–D) Cotyledons of 7-d-old plants on 0 (A, B) or 0.5 mM (C, D) phenanthrene. (E, F) Leaves of 14-d-ld plants on 0.5 mM phenanthrene. (G–N) Leaves of 21-d-old plants on 0.25 mM phenanthrene. Images show different stages of fluorescence accumulation in a trichome and spot formation. (O, P) Leaves of 28-d-old plants on 0.5 mM phenanthrene. Scale bars 10 μm, except 100 μm in (E), (F), (O), and (P).

any carrier. This view is supported by the observations of Wild *et al.* (2004), who traced the movement of anthracene (also a three-ring PAH) in maize leaves using two-photon excitation microscopy. The authors showed that anthracene, applied as droplets on leaves, formed focused nests on the leaf surface, from which it diffused into the cytoplasm within 72 h. Moreover, from experiments with two medium layers (bottom with, and top without, phenanthrene) and multiple cell Petri dishes (some cells with, some without phenanthrene), it is known that phenanthrene can enter *Arabidopsis* both through direct contact with the tissue and from the air (details not shown).

Based on the observations presented here, the development of the white spots/lesions typically starts at trichomes (Fig. 9F, H, J). After a certain amount of PAH accumulation in the trichome, it seems to diffuse to the base of the cell, and spread into the adjacent cells of the trichome basement (Fig. 9L). Finally, the trichome collapses (Fig. 9N). It is unclear if trichomes act as entering points for the PAH, 'combing' phenanthrene from the air, or if they import the PAH coming from other cell types and tissues. Notably, in these experiments trichomes never stained with trypan blue. Possibly, the trichome cells remain alive until they suddenly collapse. These observations corroborate the special role in detoxification that has been designated to trichomes in some previous studies: Trichomes of *Brassica juncea* accumulate cadmium (Salt *et al.*, 1995), and *Arabidopsis* trichomes contain at least 300-fold increased glutathione concentrations compared with other epidermal cells, suggesting that trichomes may function as an efficient site of xenobiotic conjugation (Gutierrez-Alcala *et al.*, 2000). Trichome-specific engineering with PAH-degrading enzymes may be one strategy for future phytoremediation. Many microbial PAH biodegrading enzymes are mono- and dioxygenases (Cerniglia, 1997; Kanaly and Harayama, 2000); and, therefore, it is tantalizing to think that plants

with increased levels of particular mono- or dioxygenases, especially in trichomes, will show increased tolerance and transform PAHs to non-toxic intermediate compounds.

In animal cells, PAH metabolism causes oxidative stress (Burchiel and Luster, 2001). In this regard, plant and animal cells respond similarly to PAH exposure, as shown by the production of H_2O_2 (Fig. 4) and localized cell death (Fig. 3) in *Arabidopsis*. On the other hand, in plant defence against pathogenic organisms (Lamb and Dixon, 1997; Chamnongpol *et al.*, 1998), or ozone exposure (Rao *et al.*, 2000), programmed cell death is also mediated by local increases in ROS levels. The origin of H_2O_2 under phenanthrene exposure remains unclear. It remains to be determined whether a NADPH oxidase generates the H_2O_2 or it comes from the oxidation of PAHs. In addition, it is unclear whether SA potentiates H_2O_2 generation as has been reported in plant defence responses.

PAH exposure does not induce a general shut down of metabolic activities, because the expression levels of housekeeping genes were not affected by phenanthrene treatment (Fig. 6), rather the response is more specific. The small size of plants grown on phenanthrene may be attributed to a decrease in cell division or cell expansion rate, or both. However, experiments with plants expressing the *CyclinB1;1::GUS* reporter, which enables an analysis of cell division (Colón-Carmona *et al.*, 1999), indicate that spatio-temporal patterns of mitotic activity are not affected by phenanthrene (data not shown). The reduction in expansin expression suggests that growth reduction may largely be due to an inhibition of cell enlargement. However, because *EXP8* may be expressed at higher levels in floral organs compared with leaves (http://mpss.udel.edu/at/GeneAnalysis.php?featureName=AT2G40610), the reduced expression levels of the gene may partly be explained by the delayed flowering of phenanthrene-treated plants (Figs 1G, 2C, D). The delay in flowering is remarkable, since most abiotic stress situations usually tend to accelerate flowering. An analysis of hormone levels may provide an explanation for this response.

Currently, it is unclear how PAH stress relates to other abiotic or biotic stresses. It is exciting that the marker gene of the systemic acquired resistance, *PR1*, is induced in phenanthrene-exposed plants (Fig. 6). The induction of *PR1* is regulated by SA, which mediates defence responses against biotrophic pathogens (Thomma *et al.*, 1998). However, the extent of defence gene activation upon PAH exposure remains to be determined. Results from a genome-wide analysis will provide insight into the activation of stress signalling pathways and the involvement of other hormones. Studying the crosstalk between phenanthrene stress, disease resistance, and oxidative stress pathways is important since it may be a major factor in any PAH phytoremediation strategy. For example, plants over-expressing the enzyme NahG from *Pseudomonas*, an enzyme in the naphthalene metabolic pathway, which converts SA to catechol, accumulate little SA and are more susceptible to pathogens (Delaney *et al.*, 1994). To understand the crosstalk between signalling pathways of PAH and other environmental stresses, it will be interesting to study the PAH responses of *nahG* over-expressing, or SA over-producing plants, as well as *Arabidopsis* mutants affected in the production or perception of other hormones.

In conclusion, novel and conventional stress responses to phenanthrene, an organic environmental pollutant, have been identified. Integrating these data into signalling and metabolic pathways, and applying these models towards PAH phytoremediation will expand the knowledge surrounding abiotic stress responses. The genomic tools available for *Arabidopsis* offer an excellent basis to screen for genes relevant in PAH phytoremediation. When such genes are identified, native plant species with enhanced biodegradation properties can easily be identified, as well as designing transgenic plants for PAH remediation will be successful (Cobbett and Meagher, 2002).

Acknowledgements

The *GSTF2::GUS* seeds were a kind gift from Peter Goldsbrough, Purdue University, USA. The authors thank Alejandro Calderon-Urrea, Michael Shiaris, and David Weisman for critically reading the manuscript. This work was supported by the University of Massachusetts Boston and by a Joint Interagency Program on Phytoremediation Research grant (National Science Foundation no. IBN-0343856) to AC-C, and an Undergraduate Mentoring in Environmental Biology fellowship from the National Science Foundation to TMT.

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