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RESEARCH PAPER

Microarray expression profiling of *Arabidopsis thaliana* L. in response to allelochemicals identified in buckwheat

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Abstract

Buckwheat (*Fagopyrum esculentum* Moench) is an important annual plant cultivated for grain or as a cover crop in many countries, and it is also used for weed suppression in agro-economic systems through its release of allelochemicals. Little is known, however, concerning the mode of action of allelochemicals or plant defence response against them. Here, microarrays revealed 94, 85, and 28 genes with significantly higher expression after 6 h of exposure to the allelochemicals fagomine, gallic acid, and rutin, respectively, compared with controls. These induced genes fell into different functional categories, mainly: interaction with the environment; subcellular localization; protein with binding function or cofactor requirement; cell rescue; defence and virulence; and metabolism. Consistent with these results, plant response to allelochemicals was similar to that for pathogens (biotic stress) or herbicides (abiotic stress), which increase the concentration of reactive oxygen species (ROS; with consequent oxidative stress) in plant cells. The data indicate that allelochemicals might have relevant functions, at least in part, in the cross-talk between biotic and abiotic stress signalling because they generate ROS, which has been proposed as a key shared process between these two stress mechanisms.

Key words: Abiotic stress, allelopathy, biotic stress, fagomine, *Fagopyrum esculentum* Moench, gallic acid, gene expression, microarray, mode of action, rutin.

Introduction

Allelopathy, the chemical inhibition of one plant species by another, represents a form of chemical warfare between neighbouring plants competing for limited light, water, and nutrient resources. Allelopathic interactions have been proposed to have profound effects on the evolution of plant communities through the loss of susceptible species via chemical interference and by imposing selective pressure favouring individuals resistant to inhibition from a given allelochemical (Baerson *et al.*, 2005). Most plant species, including crops, are capable of producing and releasing biologically active compounds (allelochemicals) into the environment to suppress the growth of other plants. Allelochemicals have usually been considered to be secondary metabolites or waste products of the main metabolic pathways in plants, and they do not appear to play a role in the primary metabolism essential for plant survival (Swain, 1977). Although most plants produce phytotoxic allelochemicals, relatively few, including buckwheat (*Fagopyrum esculentum* Moench), have strong allelopathic properties (Campbell, 1997; Tsuzuki, 2001; Bhowmik and Inderjit, 2003; Fujii *et al.*, 2005; Khanh *et al.*, 2005; Xuan *et al.*, 2005; Golisz *et al.*, 2007*a*).

Buckwheat is used for weed suppression in agro-economic systems through its release of allelochemicals, and it is also an important annual plant cultivated for grain or as a cover crop in many countries. Certain compounds that can inhibit the growth of other plants have been identified in buckwheat. In a previous study, Golisz *et al.* (2007*b*) identified eight allelochemicals including rutin, quercetin, (+)-catechin, (–)-epicatechin, chlorogenic acid, caffeic acid, ferulic acid, and gallic acid. The total activity of rutin, as calculated by concentration and growth inhibitory potential, suggests that this compound is the major allelochemical in buckwheat. However, Iqbal *et al.* (2007) have suggested that gallic acid might play a key role and act synergistically with minor allelochemicals in buckwheat to produce other allelopathic activities.

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Moreover, they also proposed that fagomine is a significant growth inhibitor of lettuce seedlings.

The present study employed three allelochemicals, fagomine (a piperidine alkaloid), gallic acid (a phenolic acid), and rutin (a flavonoid), which are responsible for the allelopathic potential of buckwheat (Golisz *et al.*, 2007*b*; Iqbal *et al.*, 2007). Most studies have focused on the relevance of the activities of these compounds to mammalian physiology, but not that of plants. Fagomine has been found in the aerial parts of buckwheat (Iqbal *et al.*, 2002), but also in leaves and roots of *Xanthocercis zambesiaca* (Leguminosae) (Ramalingam, 2005). Fagomine reduces the blood glucose level and increases plasma insulin levels in diabetic mice. The fagomine-induced potentiation of insulin release may contribute in part to its antihyperglycaemic action (Bnouham *et al.*, 2006). In another study, fagomine showed potent inhibitory activity toward rice α-glucosidase (Asano *et al.*, 2000). Gallic acid has been identified as an allelopathic agent in a number of studies, including those of Rudrappa *et al.* (2007) and Weidenhamer and Romeo (2004). Moreover, Abdelwahed *et al.* (2007) assessed its antimutagenic and antioxidant activities on human leukaemia cells and concluded that it acts as an antimutagen by directly influencing the activity of DNA repair enzymes by modulating their gene expression. Also, Soobrattee *et al.* (2005) reported that gallic acid is the most potent antioxidant amongst simple phenolics. Among these compounds, much more information is available on rutin because it is an important flavonoid—especially for human health—because it has antibacterial, antiviral, antihypertensive, and antioxidant activities. Moreover, flavonoids have functions that are critical to various aspects of plant life related to interactions with the environment: they protect the plant against ultraviolet radiation, they have antimicrobial properties, and they act as a deterrent for herbivores by limiting assimilation of dietary protein and inhibiting digestive enzymes (Pourcel *et al.*, 2006). Furthermore, flavonoids act as scavengers of free radicals such as reactive oxygen species (ROS) and also prevent their formation by chelating metals (Pourcel *et al.*, 2006).

Relatively little is known about the molecular mode of action of allelochemicals and the plant defence response against them (Duke *et al.*, 2005). To address such issues, *Arabidopsis thaliana* L. has been an excellent model to study plant response to allelochemicals and other environmental toxins (Baerson *et al.*, 2005). Moreover, microarray techniques have become a standard tool for genome-wide monitoring of gene expression. Hence, the present work uses microarrays to analyse gene expression profiles of *Arabidopsis thaliana* L. exposed to fagomine, gallic acid, and rutin. To the best our knowledge, this is the first study to assess gene responses to these compounds using microarrays. This study may lead to a better understanding of the mode of action of tested allelochemicals.

Materials and methods

Plants and growth condition

Seeds of *A. thaliana* L. (Col-4, source NASC N933) from LEHLE SEEDS Company (Round Rock, TX, USA) were sterilized for 1 min in 70% ethanol, following by 1% sodium hypochlorite with Tween-20 (Sigma-Aldrich Group, St Louis, MO, USA) for 6 min, and finally rinsed 10 times with distilled water. Sterilized seeds were placed on 1% solidified agar (Nacalai Tesque, Inc., Kyoto, Japan) with Murashige and Skoog Plant Salt Mixture (Wako Pure Chemical Industries, Ltd, Japan) and 1% sucrose (Wako Pure Chemical Industries) in Agripot (Iwaki, Tokyo, Japan). The seeds in Agripot were cold treated (4 °C) for 3 d in darkness and then transferred to a growth chamber. Plants were maintained in the growth chamber on a schedule of 16 h light (22 °C) and 8 h dark (20 °C) (Boyes *et al.*, 2001; Baerson *et al.*, 2005).

Allelochemical treatment

Arabidopsis plants were grown in Agripot as described above. After 20 d in the growth chamber, plants were removed from agar and transferred to solution culture in distilled water containing one of the following allelochemicals: fagomine (10 mg kg^{-1} of water), gallic acid (20 mg kg^{-1}), or rutin (200 mg kg^{-1}). These concentrations reflect specific activities (EC$_{50}$—the effective concentration of the compound to induce half-maximum inhibition) as determined in previous studies (Golisz *et al.*, 2007*b*; Iqbal *et al.*, 2007). Plants transferred to distilled water without any compounds were used as a control. After 6 h of treatment, plants were collected and immediately frozen in liquid nitrogen and then stored at –80 °C prior to analysis. Each treatment was replicated three times in each of three independent experiments.

Total RNA

The total RNA from 20-d-old *Arabidopsis* plants was isolated using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of isolated total RNA was assessed by spectrophotometry and gel electrophoresis, respectively.

Microarray data analysis

Isolated total RNA was amplified and labelled as described in the GeneChip Expression Analysis Technical Manual, Rev.5 (Affymetrix, Santa Clara, CA, USA). Total RNA (1 μg) was converted into double-stranded cDNA using the One-Cycle cDNA Synthesis kit (Affymetrix). *In vitro* transcription reactions were performed using a GeneChip IVT labelling kit, which includes T7 RNA polymerase and biotin-labelled ribonucleotides. The cDNA (15 μg) was hybridized to an Affymetrix GeneChip Arabidopsis ATH1 Genome Array. The array was incubated for 16 h, then automatically washed and stained using the GeneChip Hybridization, Wash and Stain kit (Affymetrix). The Probe Array was scanned using a GeneChip Scanner 3000 7G.

GeneSpring Viewer 5.2 software (Agilent Technologies, Santa Clara, CA, USA) was used for expression analysis. Genes were defined as responsive when transcripts were detected in at least two of three independent experiments (biological replicates) and when the signals were significantly different ($P \leqslant 0.05$) compared with control plants.

Microarray validation: quantitative real-time RT-PCR analysis

The total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was carried out using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). All kits

were used according to the manufacturer's instructions. Primers for quantitative real-time RT-PCR were designed using PRIMER EXPRESS software (v 2.0, Applied Biosystems, Foster City, CA, USA). A primer pair was used to amplify the constitutively expressed control gene encoding elongation factor 1α (EF1α; At5g60390, Becher *et al.*, 2004). The sequences of all primers are presented in Supplementary Table S1 available at *JXB* online.

Quantitative real-time RT-PCR was performed on the ABI Prism 7700 Sequence Detection System using SYBR Green. cDNA was diluted 1:50 with nuclease-free water. Reactions were in 20 μl containing 9 μl of qPCR Master Mix (Eurogentec, Liege, Belgium), 0.6 μl of SYBR Green (Eurogentec), and 0.5 μmol of forward and reverse primers (Operon Biotechnologies, Tokyo, Japan). The following standard thermal profile was used: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60°C. Each sample was analysed in three technical replicates, and the resulting data were analysed using ABI Prism 7000 SDS Software. For the calculation of the threshold cycle (C_T) values, the auto-C_T function was used. For further calculations, the mean value of each triplicate was used. To normalize the target gene expression, the difference between the C_T of the target gene and the C_T of EF1α (constitutive control) for the respective template was calculated (ΔC_T value). To calculate fold changes in gene expression, the ΔC_T value was calculated as follows: $\Delta C_T = C_T$ (target gene)–C_T (constitutive control gene). Relative transcript levels were calculated as: $1000 \times 2^{-\Delta CT}$.

Results

Microarray analysis demonstrates that *A. thaliana* plants respond to each of the three allelochemicals added to the culture medium by changes in gene expression. Culturing for 6 h caused up-regulation (≥2-fold) of 94 genes for fagomine, 85 for gallic acid, and 28 for rutin, compared with the control. Among these genes, seven were common for all three compounds, 23 for fagomine and rutin, nine for fagomine and gallic acid, and eight for rutin and gallic acid (Fig. 1). Supplementary Table S2 at *JXB* online presents all the genes that were significantly (P ≤0.05) up-regulated upon exposure to each of the three allelochemicals.

The seven common genes up-regulated by ≥2-fold by fagomine, gallic acid, and rutin were as follows: ankyrin repeat family protein (At4g14400/245265_at), disease resistance protein (At4g16890/245451_at), pathogenesis-related protein (At2g14610/266385_at), glutathione *S*-transferase (GST) (At1g78370/260745_at), and three expressed proteins of unknown function (At1g17870/255891_at; At3g22231/256766_at; At2g32160/265698_at) (Fig. 1, Table 1).

The 26 genes up-regulated by ≥2-fold by two or three individual compounds (Table 1) were distributed into 14 different functional categories (Fig. 2) based on FunCat assignments available through the MIPS *A. thaliana* database (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). The most populated categories were 'interaction with the environment' and 'subcellular localization', representing 33% and 29%, respectively, of all functions
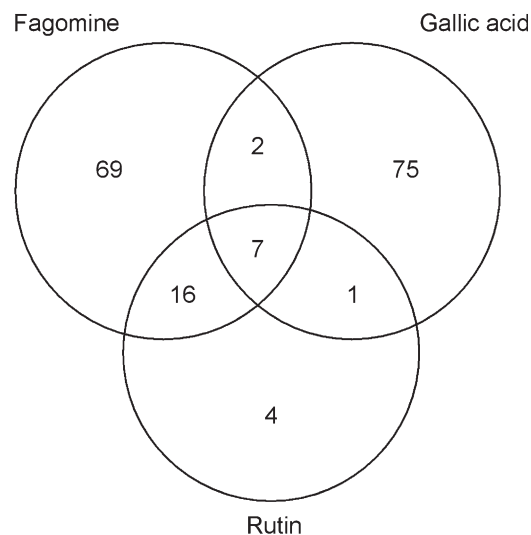

Fig. 1. Venn diagrams displaying *Arabidopsis thaliana* genes significantly (P ⩽ 0.5) induced by ⩾2-fold (normalized) after 6 h of exposure to fagomine, gallic acid, or rutin in the aquaculture medium. Seven common genes were up-regulated by each tested compound (representative public ID/probe set identifier): ankyrin repeat family protein (At4g14400/245265_at), disease resistance protein (At4g16890/245451_at), pathogenesis-related protein (At2g14610/266385_at), glutathione *S*-transferase (At1g78370/260745_at), and three expressed proteins (At1g17870/255891_at; At3g22231/256766_at; At2g32160/265698_at).

assigned. Also noteworthy are the categories of 'protein with binding function or cofactor requirement' and 'cell rescue, defence and virulence', representing 25%, or genes associated with 'systemic interaction with the environment' and 'metabolism', representing 21% of all functions assigned.

Gallic acid, which up-regulated 20 genes by ≥3-fold, was a relatively more potent allelochemical in this regard than fagomine (13 genes) and rutin (six genes) (Table 2). The highest measured fold change (15.57) was with gallic acid for the gene encoding the class II heat shock protein (At5g12030). Among the 20 genes highly up-regulated by gallic acid, eight encode heat shock proteins (Table 2). Other gallic acid-induced genes of interest were leucine-rich repeat protein, chitinase, and ubiquinol–cytochrome *c* reductase complex (Table 2), transcription factors WRKY and MYB15, and Cu–Zn superoxide dismutase, peroxidase, and cytochrome P450 (Supplementary Fig. S2 at *JXB* online). Some of these gene products are involved in generating ROS during oxidative stress. In the case of fagomine treatment, lipid transfer protein (LTP; At3g08770) had the highest fold-induction of 7.45, whereas in response to rutin this gene was induced only by 3.38-fold (Table 2). Moreover, fagomine and rutin induced nicotianamine synthase (At5g56080) expression by 3.72- and 3.12-fold, respectively (Table 2).

The Genevestigator online search tool Meta-profile analysis (https://www.genevestigator.ethz.ch/) reports expression as a heat map where a colour spectrum defines

Table 1. *The 26 common* Arabidopsis thaliana *genes significantly (P ≤ 0.05) induced by ≥2-fold (normalized) by at least two of the allelochemicals fagomine, gallic acid, and rutin after 6 h of exposure of plants to one allelochemical in the aquaculture medium*

ID_Affymetrix	Fold-change			Gene title_Affymetrix
	Fagomine	Gallic acid	Rutin	
At4g14400	3.44	2.27	3.56	Ankyrin repeat family protein
At4g16890	2.07	3.72	2.98	Disease resistance protein (TIR-NBS-LRR class), putative
At1g17870	3.17	2.77	2.71	Expressed protein
At3g22231	2.12	2.12	2.59	Expressed protein
At1g78370	3.99	2.04	2.00	Glutathione *S*-transferase, putative
At2g32160	3.09	2.00	2.53	Expressed protein
At2g14610	4.06	2.25	2.98	Pathogenesis-related protein 1 (PR-1)
At2g15120	2.31	2.39		Pseudogene, disease resistance family protein/fatty acid elongase-related
At2g32570	2.30	2.31		F-box family protein
At5g04190	2.32		2.51	Phytochrome kinase substrate-related
At5g59680	2.52		2.45	Leucine-rich repeat protein kinase, putative
At5g56080	3.72		3.12	Nicotianamine synthase, putative
At3g62040	3.36		2.17	Haloacid dehalogenase-like hydrolase family protein
At3g56585	2.00		2.31	Hydroxyproline-rich glycoprotein family protein
At4g08780	2.19		2.55	Peroxidase, putative
At1g48750	2.45		2.00	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
At3g08770	7.45		3.38	Lipid transfer protein 6 (LTP6)
At1g21250	2.20		2.82	Wall-associated kinase 1 (WAK1)
At1g64360	2.67		2.18	Expressed protein
At1g74670	2.87		2.88	Gibberellin-responsive protein, putative
At1g24020	2.29		2.02	Bet v I allergen family protein
At2g17580	2.44		2.00	Polynucleotide adenylyltransferase family protein
At2g21050	2.26		2.05	Amino acid permease, putative
At2g28780	3.51		2.00	Expressed protein
At3g21720	2.79		2.83	Isocitrate lyase, putative
At2g43620		3.19	2.64	Chitinase, putative

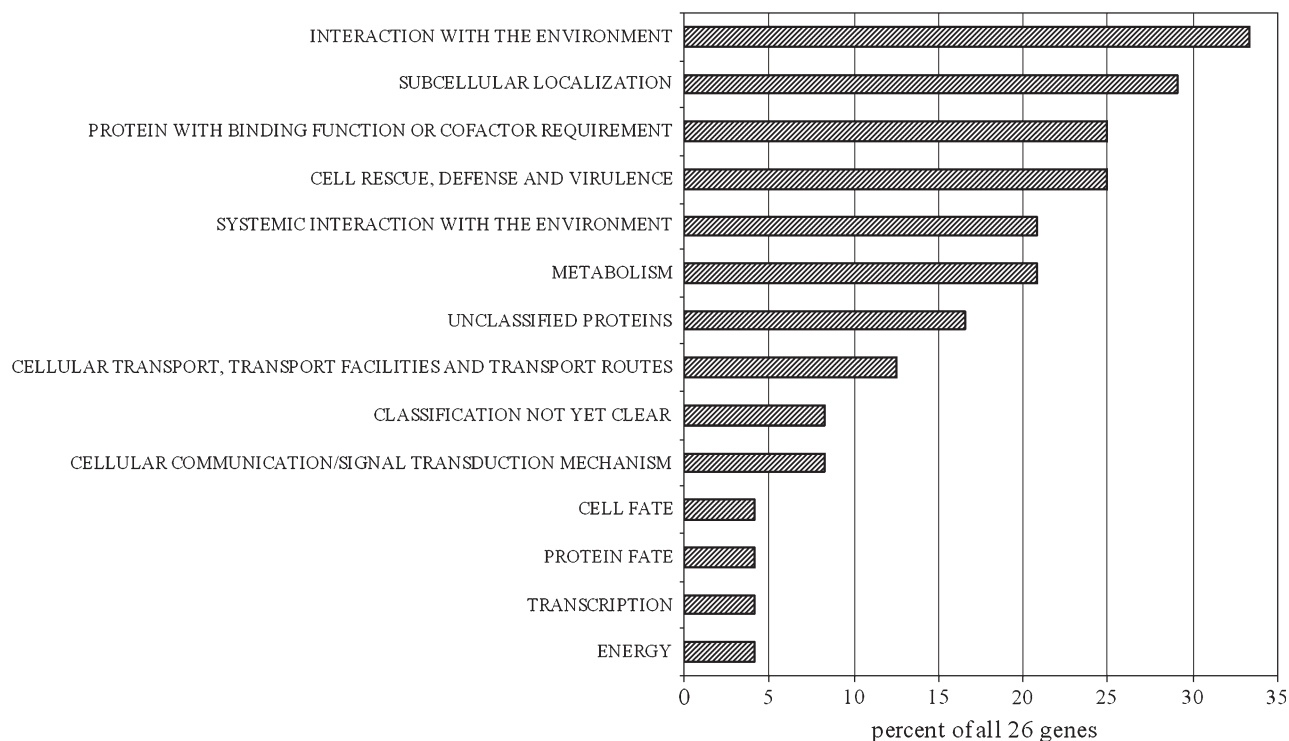

Fig. 2. Functional categorization of 26 *Arabidopsis thaliana* genes induced by ≥2-fold (normalized) by at least two of the three allelochemicals fagomine, gallic acid, and rutin (these genes are listed in Table 1). Genes were categorized based on the FunCat scheme devised by the Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/funcatDB/search_main_frame.html).

Table 2. Arabidopsis thaliana *genes significantly (P≤0.05) induced ≥3-fold (normalized) after 6 h of exposure of plants to fagomine, gallic acid, or rutin in the aquaculture medium*

ID_Affymetrix	Gene title_Affymetrix	Fold-change	*P*-value
	Fagomine		
At3g08770	Lipid transfer protein 6 (LTP6)	7.45	0.02
At2g14610	Pathogenesis-related protein 1 (PR-1)	4.06	0.05
At1g78370	Glutathione *S*-transferase, putative	3.99	0.03
At4g02290	Glycosyl hydrolase family 9 protein	3.95	0.02
At5g56080	Nicotianamine synthase, putative	3.72	0.05
At2g28780	Expressed protein	3.51	0.01
At4g14400	Ankyrin repeat family protein	3.44	0.02
At1g78970	Lupeol synthase (LUP1)/2,3-oxidosqualene-triterpenoid cyclase	3.41	0.01
At3g62040	Haloacid dehalogenase-like hydrolase family protein	3.36	0.05
At1g17870	Expressed protein	3.17	0.05
At1g52400	Glycosyl hydrolase family 1 protein/beta-glucosidase, putative (BG1)	3.15	0.01
At2g32160	Expressed protein	3.09	0.01
At1g32450	Proton-dependent oligopeptide transport (POT) family protein	3.00	0.00
	Gallic acid		
At5g12030	17.7 kDa class II heat shock protein 17.6A (HSP17.7-CII)	15.57	0.02
At5g12020	17.6 kDa class II heat shock protein (HSP17.6-CII)	10.33	0.02
At3g46230	17.4 kDa class I heat shock protein (HSP17.4-CI)	6.57	0.02
At2g02010	Glutamate decarboxylase, putative	5.91	0.05
At5g48570	Peptidyl-prolyl *cis-trans* isomerase, putative/FK506-binding protein, putative	5.58	0.03
At5g52640	Heat shock protein 81-1 (HSP81-1)/heat shock protein 83 (HSP83)	5.36	0.02
At3g12580	Heat shock protein 70, putative/HSP70, putative	5.08	0.03
At2g29500	17.6 kDa class I small heat shock protein (HSP17.6B-CI)	4.63	0.05
At1g74310	Heat shock protein 101 (HSP101)	4.53	0.01
At1g54050	17.4 kDa class III heat shock protein (HSP17.4-CIII)	4.22	0.01
At4g22610	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	3.86	0.01
At5g09530	Hydroxyproline-rich glycoprotein family protein	3.73	0.03
At4g16890	Disease resistance protein (TIR-NBS-LRR class), putative	3.72	0.01
At2g39200	Seven transmembrane MLO family protein (MLO12)	3.56	0.05
At1g51800	Leucine-rich repeat protein kinase, putative	3.47	0.03
At1g02430	ADP-ribosylation factor, putative	3.29	0.05
At5g25450	Ubiquinol–cytochrome *c* reductase complex 14 kDa protein, putative	3.11	0.03
At3g02240	Expressed protein	3.10	0.03
At5g55050	GDSL-motif lipase/hydrolase family protein	3.05	0.03
At3g54420	Class IV chitinase (CHIV)	3.00	0.04
	Rutin		
At4g14400	Ankyrin repeat family protein	3.56	0.01
At3g08770	Lipid transfer protein 6 (LTP6)	3.38	0.05
At3g49870	ADP-ribosylation factor, putative	3.35	0.05
At5g56080	Nicotianamine synthase, putative	3.12	0.05
At2g14610	Pathogenesis-related protein 1 (PR-1)	3.00	0.05
At4g16890	Disease resistance protein (TIR-NBS-LRR class), putative	3.00	0.04

the relative expression of each gene. Querying the database using a list of seven common genes that were up-regulated by each of the three tested allelochemicals tentatively identified the developmental tissues (Fig. 3) in which these compound-responsive genes are expressed during the normal life cycle of *Arabidopsis*. These seven common genes are preferentially expressed in seedling cotyledons, the inflorescence pedicel and cauline leaf, and the rosette senescent leaf; expression is particularly high for disease resistance protein (At4g16890), ankyrin repeat protein (At4g14400), and an expressed protein (At2g32160) (Fig. 3).

Figure 4 presents Genevestigator compilation data for stimulus-responsive expression of gene clusters for which the data are consistent across many microarray experiments. These compilations were derived from independent experiments that may have used different stimulus durations and/or application methods. Thus, these heat maps represent averages of behaviour and are colour coded by the average expression level change between stimulated and unstimulated conditions. Among seven common genes up-regulated by all three allelochemicals, pathogenesis-related protein (At2g14610) is one of most highly regulated genes; the most potent stimuli affecting the expression of this gene are organisms that elicit biotic stress, such as *Erysiphe orontii* or *Phytophthora infestans*, and abiotic stresses such as wounding, cold, and osmotic shock (Fig. 4). The gene encoding expressed protein At1g17870 is also up-regulated by abiotic stress such as heat, osmotic shock, salt stress, or exposure to abscisic acid. Four genes were up-regulated upon stimulation with the plant hormone, salicylic acid: pathogenesis-related protein (At2g14610), ankyrin (At4g14400), and two expressed proteins (At3g22231, At2g32160). Among the
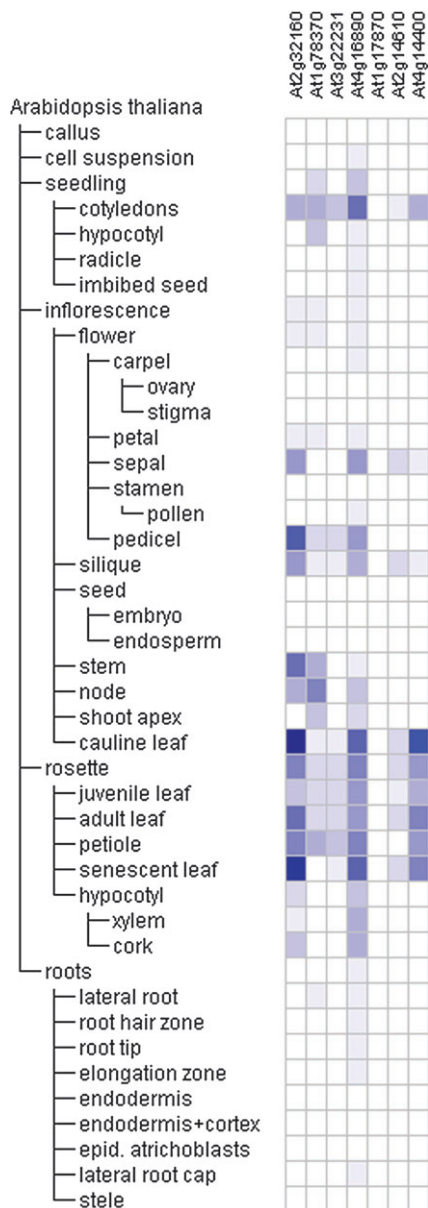
Fig. 3. Heat map showing the levels of *Arabidopsis* gene expression throughout one plant life cycle. The *Meta-Profile Analysis* tool of the Genevestigator software (https://www.genevestigator.ethz.ch/) was utilized with a probe set for seven genes up-regulated by all three allelochemicals. The blue–white scheme was chosen by the software developers to visualize gene expression levels and discern slight changes in signal intensity. The colour/intensity for each gene expression profile was normalized such that the highest signal intensity was defined as 100% (dark blue), and the absence of signal was defined as 0 (white).

seven common genes identified, the disease resistance protein (At4g16890) was up-regulated to a lesser degree based on this analysis (Fig. 4).

To verify the microarray results, quantitative real-time RT-PCR assays were carried out for the seven common genes that were up-regulated by fagomine, gallic acid, and rutin (Fig. 5). In general, the two methods gave similar results in terms of fold change, and all genes were shown to be up-regulated. In the case of rutin, real-time RT-PCR yielded considerably higher transcript levels compared with the microarray results (Fig. 5), suggesting that rutin is the most effective of the three tested compounds, in agreement with a previous study using another method (Golisz *et al.*, 2007*b*). Relative transcript levels in plants exposed to fagomine or gallic acid were rather similar in both methods, although the levels for both compounds were lower compared with rutin. Among the tested genes, expressed protein (At1g17870) had the highest expression level for rutin and gallic acid with respect to the control plants, and for fagomine, it was GST (At1g78370) (Fig. 5). Conversely, the lowest fold increases was for expressed protein (At3g22231) for rutin and gallic acid, and another expressed protein (At1g17870) for fagomine compared with the control expression level (Fig. 5).

Discussion

The role of allelopathy in weed management has gained a great deal of attention over the last two decades (Wu *et al.*, 1999; Bertholdsson, 2005). Weed suppression by crop allelopathy during the early establishment period could reduce the need for early-season application of commercial herbicides, with late-season weed control provided by the relative competitiveness of crops over weeds. The genetic enhancement of crop allelopathy for weed management has been demonstrated (Duke *et al.*, 2001). Recent reports have highlighted the response of the *Arabidopsis* transcriptome upon exposure of plants to allelochemicals such as (–)-catechin (Bais *et al.*, 2003) and benzoxazolin-2(3H)-one (BOA) (Baerson *et al.*, 2005).

Plant response to allelochemicals, according to these microarray results, may be caused by reactions that are similar to those occurring during biotic and/or abiotic stress. Furthermore, a few papers have suggested crosstalk between abiotic and biotic stress response pathways

Fig. 4. Heat map showing the levels of gene expression in response to external stimuli. The *Meta-Profile Analysis* tool of the Genevestigator software (https://www.genevestigator.ethz.ch/) was utilized with a probe set for seven genes up-regulated by all three allelochemicals as follows, from the left side: At2g32160, At1g78370, At3g22231, At4g16890, At1g17870, At2g14610, and At4g14400. The heat map displays the results for a given list of genes, with colour coding representing relative expression values. Columns represent probe sets, and rows represent stimuli. All gene expression profiles were normalized for colour from red through black to green. Red and green indicate relatively higher and lower expression levels, respectively, upon stimulation. Black indicates no change between conditions.
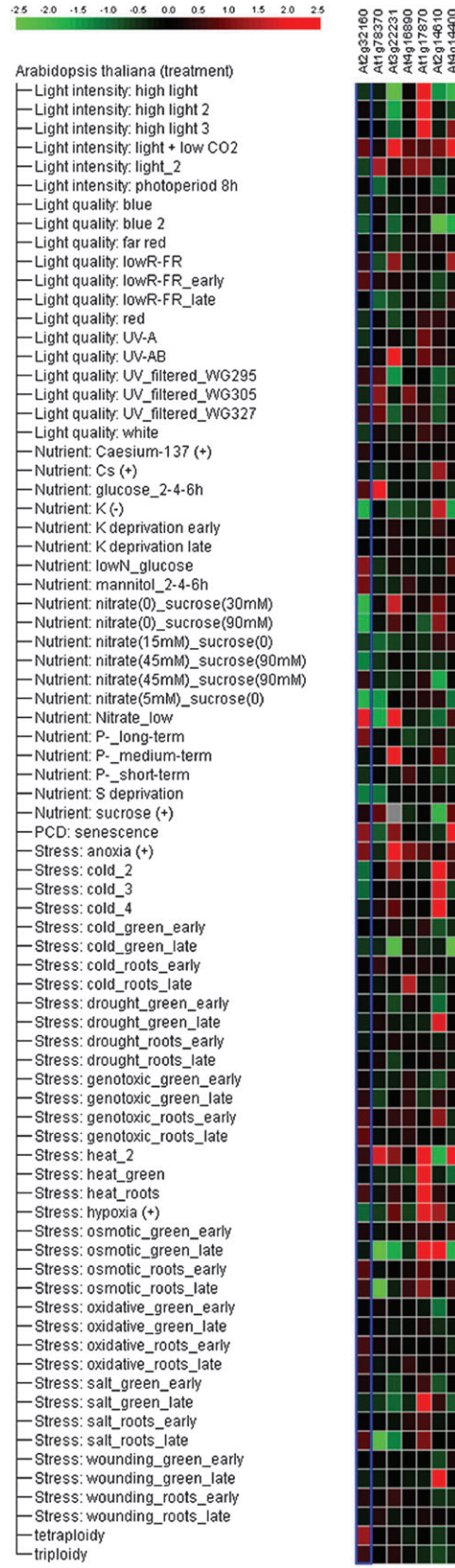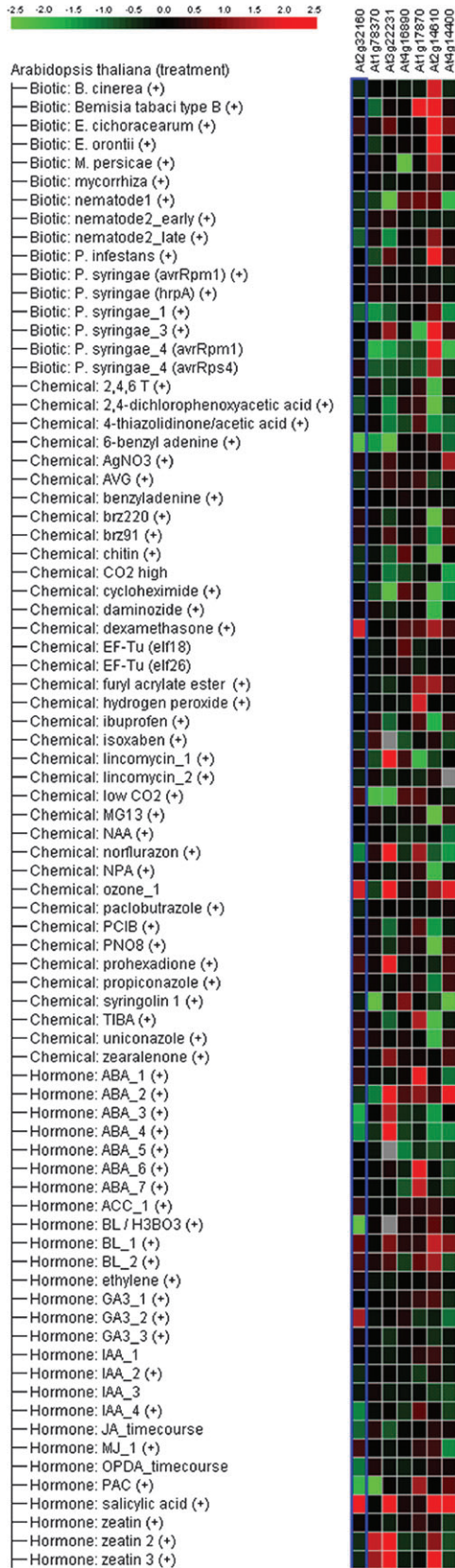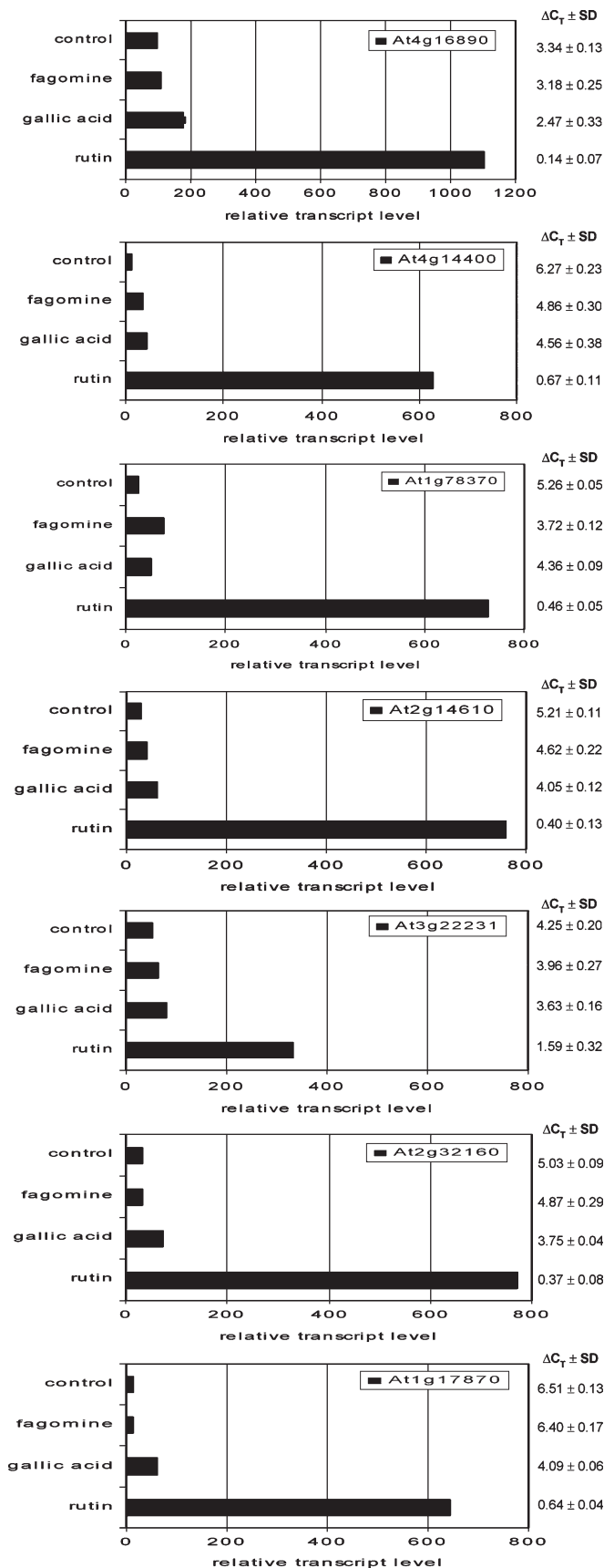
(Narusaka *et al.*, 2004; Fujita *et al.*, 2006) as well as cross-talk among various signalling pathways under each type of stress (Knight and Knight, 2001; Chinnusamy *et al.*, 2004; Taylor *et al.*, 2004; Kaur and Gupta, 2005). Biotic and abiotic stresses regulate the expression of different but overlapping sets of genes. The generation of ROS has been proposed as a key process shared between biotic and abiotic stress responses (Apel and Hirt, 2004; Narusaka *et al.*, 2004; Fujita *et al.*, 2006). Notably, several ROS-related gene products, such as ubiquinol–cytochrome *c* reductase complex, Cu–Zn superoxide dismutase, peroxidase, and cytochrome P450, were up-regulated in plants exposed to gallic acid in this study (Supplementary Table S2 at *JXB* online). These results thus suggest that gallic acid which is a potent antioxidant (Soobrattee *et al.*, 2005) generated elevated levels of ROS. This is somewhat counterintuitive, but it is worth noting and is in agreement with other reports that antioxidant compounds such as gallic acid (Rudrappa *et al.*, 2007) and (±)-catechin (Prithiviraj *et al.*, 2007) increased production of ROS in *Arabidopsis* plants.

Data from these studies show that brief exposures to low doses of fagomine, gallic acid, and rutin result in a significant genetic response in target plants. This implies that these compounds may exert a significant effect on the growth of target plants even though they are present at low concentration. However, it is important to note that the phytotoxic activity of allelochemicals in natural ecosystems and agroecosystems is affected by many factors through their effects on the behaviour in soil and both the donor and receiver plants (Kobayashi, 2004).

The common genes that were up-regulated by each of fagomine, gallic acid, and rutin (see Table 1, top) have been described as stress-related genes—some of which have been well characterized in this regard. For example, ankyrin repeats form a domain in accelerated cell death 6 (ACD6), overexpression of which confers enhanced disease resistance by priming stronger and quicker defence responses during pathogen infection or plant development, or upon treatment with an antagonist of the key defence regulator salicylic acid (Lu *et al.*, 2005). The best characterized ankyrin protein from plants is NPR1/

Fig. 5. Real-time RT-PCR analysis of expression of seven common genes: disease resistance protein (At4g16890), ankyrin repeat family protein (At4g14400), glutathione *S*-transferase (At1g78370), pathogenesis-related protein (At2g14610), and three expressed proteins (At3g22231, At2g32160, At1g17870) in *Arabidopsis thaliana*. Transcript levels were assessed by real-time RT-PCR in plants following 6 h of exposure of 20-d-old plants to fagomine, gallic acid, or rutin in the aquaculture medium. Values are mean $\Delta CT \pm SD$, and the relative transcript level was calculated from three technical replicates. The ΔCT values were calculated as follows: ΔCT (target gene) = CT (target gene)–CT (constitutive control gene, EF1α), whereby the CT value is the cycle number at which the PCR product exceeded a set threshold. Relative transcript levels were calculated as: $1000 \times 2^{-\Delta CT}$.

NIM1, which is involved in salicylic acid-dependent disease resistance and in a salicylic acid-independent resistance response elicited by certain root-associated bacteria (Lu *et al.*, 2003). GST, another common up-regulated protein in this study, appears to be ubiquitous in plants and has defined roles in herbicide detoxification (Crawford *et al.*, 2000; Dixon *et al.*, 2002; Wagner *et al.*, 2002; Blokhina *et al.*, 2003). The primary biochemical function of many GSTs is conjugation, either of xenobiotics or of intermediates and secondary metabolites. In addition, certain GSTs play roles as peroxidases or in regenerating ascorbate from dehydroascorbate (Foyer and Noctor, 2005). Pathogenesis-related protein (At2g14610) is induced in response to salicylic acid and a variety of pathogens. Among three proteins up-regulated by all three allelochemicals, one (At3g22231) is distinct because its expression is regulated by the circadian clock and it is up-regulated in response to both virulent and avirulent strains of *Pseudomonas syringae* pv. tomato (Sauerbrunn and Schlaich, 2004). A second protein (At1g17870) contains transmembrane helices near its C-terminus and has a conserved zinc-binding motif, and it is involved in response to abiotic stresses such as heat and high-intensity light (Chen *et al.*, 2005). The function of a third protein (At2g32160) is unknown. Further annotation of this protein, however, may provide important clues toward understanding the mode of action of allelochemicals.

Among genes up-regulated by $\geqslant$3-fold by each of the allelochemicals of interest was heat shock protein induced by gallic acid (Table 2). These proteins are expressed in plants not only when they experience high temperature stress but also in response to a wide range of other environmental insults, such as water stress, salinity, osmotic stress, and cold and oxidative stress (Grover *et al.*, 2001; Wang *et al.*, 2004). Heat shock proteins/ chaperones play a crucial role in protecting plants against stress and in the re-establishment of cellular homeostasis by binding to and sequestering misfolded or unfolded proteins. Moreover, they also act synergistically with other cellular stress response pathways to limit cellular damage (Wang *et al.*, 2004). In the case of fagomine and rutin, of interest was LTP6 and nicotianamine synthase. LTPs have been suggested to participate in cutin assembly and in plant defence against pathogens (Arondel *et al.*, 2000). Nicotianamine synthase is a highly important enzyme for the precise regulation of the iron metabolism that is required for the control of basic cellular processes such as electron transport in photosynthesis and respiration (Herbik *et al.*, 1999), and it is also a cytosolic chelator of Zn^{2+} (Trampczynska *et al.*, 2006).

The present data suggest that allelochemicals such as fagomine, gallic acid, and rutin cause reactions in plants that are similar to those involved in response to pathogens or herbicides—environmental factors that increase ROS levels inside plant cells, which results in oxidative stress.

Oxidative stress may arise from any abiotic or biotic stress that generates ROS (Bray *et al.*, 2000; Blokhina *et al.*, 2003). ROS include not only free radicals (superoxide radical and hydroxyl radical), but also molecules such as hydrogen peroxide, singlet oxygen, and ozone (Blokhina *et al.*, 2003; Mittler *et al.*, 2004). Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by various cellular enzymatic and non-enzymatic mechanisms (Apel and Hirt, 2004). ROS control and regulate many different processes in plants, such as growth, cell cycle, programmed cell death, pathogen defence, hormone signalling, stomatal behaviour, development, and biotic and abiotic stress responses (Apel and Hirt, 2004; Mittler *et al.*, 2004; Laloi *et al.*, 2007). Under steady-state conditions, ROS are scavenged by various antioxidative defence mechanisms (Laloi *et al.*, 2007). However, under stress conditions, ROS have been proposed to affect stress responses in two different ways. First, they may react with a large variety of biomolecules, and thus cause irreversible damage that can lead to tissue necrosis and ultimately death of plants. On the other hand, ROS influence the expression of a number of genes and signal transduction pathways. The cells have evolved strategies to utilize ROS as environmental indicators and biological signals that activate and control various genetic stress response programmes (Apel and Hirt, 2004). It is also known that when plants are exposed to various abiotic stress conditions, a large part of the stress-induced transient increase in ROS concentration takes place within chloroplasts, when the balance between light absorption and the use of light energy is disturbed and excess light energy will lead to the inhibition of photosynthesis (Laloi *et al.*, 2006, 2007).

The present results with *Arabidopsis*, especially when plants were treated by gallic acid, suggest that environmental factors should be added to the category of allelochemicals/allelopathy. The authors assume that a comparative analysis might reveal valuable information that otherwise may not be obtainable by identifying responses that are shared (or different) across species. However, these results revealed that most of these genes are up-regulated during oxidative stress; these include Cu–Zn superoxide dismutase, calmodulin kinase, GST, heat-shock proteins, and transcription factors (WRKY and myb). Detoxification enzymes such as GST, catalases, superoxide dismutase, and ascorbate peroxidases are involved in protection from reactive singlet oxygen species; moreover, proteins involved in regulatory functions and in signal transduction, including various protein kinases and transcription factors, have a broader role in governing stress responses (Grover *et al.*, 2001). On the other hand, the allelochemicals used in this study also affected the expression of genes that are regulated in response to pathogen attack or wounding—for example, pathogenesis-related protein, disease resistance protein,

and leucine-rich repeat protein. Notably, plant hormones such as nitric oxide and salicylic acid are key regulators of responses to pathogen attack or wounding, and also of the activation of programmed cell death (Mittler, 2002; Zottini *et al.*, 2007). Defence-related stress may also induce cell death.

In conclusion, the results of this study show that allelopathy may involve genes that are up-regulated during biotic and/or abiotic stresses. This suggests that the plant pathways involved in response to allelochemicals may be shared between abiotic and biotic stresses. It is clear, however, that future studies are needed to understand better the mode of action of allelochemicals in plants with regard to their potential contribution to the biological control of weeds.

Supplementary data

The following supplementary data can be found on *JXB* online.

Table S1. Primers used for quantitative real-time RT-PCR assays.

Table S2. *Arabidopsis thaliana* genes significantly ($P \leqslant 0.05$) up-regulated ($\geqslant$2-fold) after 6 h of exposure to fagomine, gallic acid, or rutin in the aquaculture medium.

Acknowledgements

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