Low environmentally relevant levels of bioactive xenobiotics and associated degradation products cause cryptic perturbations of metabolism and molecular stress responses in Arabidopsis thaliana

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Abstract

Anthropic changes and chemical pollution confront wild plant communities with xenobiotic combinations of bioactive molecules, degradation products, and adjuvants that constitute chemical challenges potentially affecting plant growth and fitness. Such complex challenges involving residual contamination and mixtures of pollutants are difficult to assess. The model plant Arabidopsis thaliana was confronted by combinations consisting of the herbicide glyphosate, the fungicide tebuconazole, the glyphosate degradation product aminomethylphosphonic acid (AMPA), and the atrazine degradation product hydroxyatrazine, which had been detected and quantified in soils of field margins in an agriculturally intensive region. Integrative analysis of physiological, metabolic, and gene expression responses was carried out in dose–response experiments and in comparative experiments of varying pesticide combinations. Field margin contamination levels had significant effects on plant growth and metabolism despite low levels of individual components and the presence of pesticide degradation products. Biochemical and molecular analysis demonstrated that these less toxic degradation products, AMPA and hydroxyatrazine, by themselves elicited significant plant responses, thus indicating underlying mechanisms of perception and transduction into metabolic and gene expression changes. These mechanisms may explain observed interactions, whether positive or negative, between the effects of pesticide products (AMPA and hydroxyatrazine) and the effects of bioactive xenobiotics (glyphosate and tebuconazole). Finally, the metabolic and molecular perturbations induced by low levels of xenobiotics and associated degradation products were shown to affect processes (carbon balance, hormone balance, antioxidant defence, and detoxification) that are likely to determine environmental stress sensitivity.

Key words: Arabidopsis thaliana, gene expression, metabolite profiling, pesticides, pollutants, signalling pathways, stress responses, toxicants, transcript profiling, xenobiotic interactions.

Introduction

Phytochemical crop protection leads to the release of xenobiotic molecules into the environment by volatilization, spray drift, runoff, leaching, and absorption. The resulting pollution of soil and water frequently consists of mixtures of pesticides (fungicides, insecticides, and herbicides) associated with related degradation products and adjuvants. This anthropogenic and chemical pollution can affect growth and fitness of non-target organisms, with varying toxicity that depends
on biodisponibility and surrounding physico-chemical factors. Living organisms are thus more often exposed to cocktails of chemical compounds rather than individual substances. Combinations of chemical pollution have complex biological effects that are difficult to predict from the effects of single contaminants (Dévier et al., 2011). Whereas a given chemical can have negligible biological effects, its presence within a complex mixture can result in additive and deleterious effects or antagonistic effects relative to other contaminants. Triazine herbicides can increase the toxicity of organophosphate insecticides or decrease the toxicity of the fungicide prochloraz on human health (Hernández et al., 2012). The toxicity of a mixture of pesticides also involves the effects of derived degradation products. The broad-spectrum non-selective herbicide glyphosate and its main degradation product, aminomethylphosphonic acid (AMPA), are often found in freshwater ecosystems (Székács and Darvas, 2012). Glyphosate affects plants by blocking the shikimate pathway of synthesis of aromatic amino acids (Steinrücken and Amrhein, 1980), which does not exist in animal cells. Nevertheless, glyphosate at very low subagricultural concentrations elicits apoptosis in human cells, and the presence of AMPA dramatically enhances these toxic effects (Benachour and Séralini, 2009). Although described as less toxic, AMPA has been reported to be phytotoxic for soybean (Glycine max) and canola (Brassica napus) through unknown mechanisms (Reddy et al., 2004; Nandula et al. 2007).

In the context of combinations of agricultural pollution, the implantation of vegetated filter strips at the edge of agricultural fields is used to attenuate the potential environmental risk of diffuse complex mixtures of pesticides by decreasing their concentration in the surface water and groundwater (Marshall and Moonen, 2002; Reichenberger et al., 2007). As sessile organisms, plant communities are the direct targets of residual agricultural pollution, whether air-borne, water-borne, or accumulated in soils. However, few studies have dealt with plant responses to complex runoff pollution and plant buffering capacity. Most studies have determined the properties of the vegetated filter strips in terms of physical barriers, and very few consider plant communities as an active and purifying compartment (Zhang et al., 2010). Lin et al. (2011) have demonstrated that the presence of vegetated filter strips reduces soil transport of pesticides such as atrazine, metolachlor, and glyphosate, whether in dissolved form or sediment bound. Pätzold et al. (2007) showed that a 12 m wide grassed strip was highly effective for watercourse protection against metolachlor, terbuthylazine, and pendimethalin pollution. The study of plant behaviour towards pollutants in situations of environmental residual and complex contamination is therefore an important step to evaluate pesticide toxicity at low concentration and a plant’s impact on pesticide levels. Elsaesser et al. (2011) have used a toxic unit approach to assess the ability of plants to reduce the toxic effects of pollutant mixtures. The presence of vegetation in wetlands was shown to be a dominant factor in the reduction of the calculated toxicity of substances (Elsaesser et al., 2011). However, as mentioned above, this approach could not assess the impact of complex toxicant mixtures.

It is thus essential to understand the mechanisms involved in plant responses to environmentally relevant complex mixtures of contaminants. In the present work, the characterization of these mechanisms was carried out in the model plant Arabidopsis thaliana whose genome has several hundred protein-encoding genes involved in pesticide responses (Cobbett and Meagher, 2002; Ramel et al., 2007, 2009b). Arabidopsis thaliana seedlings were confronted with levels of residual complex contamination that were shown to be characteristic of field margins. The integrative analysis of the physiological, biochemical, and molecular responses revealed cryptic effects of residual pollutant levels and of their combination. The identification of cryptic effects indicates that modelling and prediction of environmental impacts of residual contamination must be undertaken with caution and that further experimental studies on the effects of contaminant mixtures and residual contamination must be carried out.

Materials and methods

Plant material and growth conditions

Seeds of A. thaliana (Columbia ecotype, Col-0) were surfaced sterilized in bayrocichlor ether (1:1, v/v), rinsed in absolute ethanol, and dried overnight. Germination and growth were carried out under axenic conditions in square Petri dishes. After seeds were sown, Petri dishes were placed in the dark at 4°C for 72 h in order to break dormancy and homogenize germination, and were then transferred to a control growth chamber at 22 °C/20 °C under a 16/8 light (6000 lux)/8 dark regime. Growth medium consisted of 0.8% (w/v) agar in Hoagland basal salt mix (H2395, Sigma-Aldrich) adjusted to pH 6. Direct exposure to pesticides during early development was carried out by seed sowing on pesticide-containing growth medium. Developmental and physiological parameters were measured after 14 d of growth. Transfer experiments were also carried out in order to mimic conditions of exposure of seedlings to shock at the same stage of photosynthetic development. After 11 d of growth under optimal conditions, seedlings were transferred to fresh growth medium containing pesticides. Developmental and physiological parameters were measured every 24 h for up to 96 h. For the analysis of gene expression and metabolite profiling, seedlings were harvested 72 h after transfer. Different pesticide treatments were tested: the broad-spectrum herbicide glyphosate (G), its degradation product aminomethylphosphonic acid (AMPA; A), the atrazine-derived degradation product hydrox atrazine (H), the fungicide tebuconazole (T), and combinations thereof.

Soil sampling and pesticide analysis

Soil samples were collected in December 2007 from the top 30 cm layer in field margins in the long-term ecological research site ‘Zone Atelier Armorique’ (ZAA) (48°36’N, 1°32’W, Brittany, France). After collection, samples were transported on ice to the laboratory, homogenized, sieved at 4 mm, and sent on ice to the CGI (Centre de Génie Industriel) analytical platform (Ploemeur, Brittany, France) for analysis. Each soil sample was analysed for the presence and quantification of a 41 molecule panel of pesticides and pesticide degradation products using high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS), gas chromatography/MS (GC/MS), or HPLC/fluorescence quantification methods (Supplementary Table S1 available at JXB online).

Analysis of growth and photosynthetic parameters

The length of the primary root was measured on vertical plates. Pigments were extracted by grinding shoots of seedlings in 80% (v/v) acetone, and the absorbance of the resulting extracts was measured at three wavelengths: 663, 646, and 470 nm. Levels of chlorophylls and total carotenoids (xanthophylls and carotenes) in these extracts were
determined from the equations given by Lichtenhailer and Wellburn (1983). Chlorophyll fluorescence and maximum photosystem II (PSII) efficiency ($F_v/F_m$) were measured using a PAM chlorophyll fluorometer system (Heinz Walz, Effeltrich, Germany) in saturating pulse mode. After dark adaptation, the maximum fluorescence of leaves ($F_m$) was measured under a subsequent saturating pulse of light. Minimum fluorescence ($F_o$) was determined under weak light before a pulse, and variable fluorescence ($F_v = F_m - F_o$) was determined.

**Metabolite profiling**

After transfer, whole seedlings were ground in liquid nitrogen to extract metabolites. The powder obtained was suspended in 600 µl of methanol:chloroform solution (2:1, v/v) and mixed for 1 min 30 s. Extracts were placed for 10 min at −20 °C, and then 400 µl of cold water were added. Samples were mixed vigorously for 1 min, and centrifuged twice for 5 min at 4000 g (4 °C). A 300 µl aliquot of the upper aqueous phase containing polar metabolites was transferred to new chromatographic glass vials, and vacuum-dried. Dry extracts were re-suspended in 30 µl of 20 mg l$^{-1}$ methoxamine–pyridine solution, and placed under automatic orbital shaking at 40 °C for 1 h. A 30 µl aliquot of N-methyl-N-trimethylsilyl trifluoroacetamide was added and derivatization was conducted at 40 °C for 1 h under agitation. All the derivatization process was automatized using a CTC CombiPal autosampler (GERSTEL GmbH and Co.KG, Mülheim an der Ruhr), ensuring an identical derivatization time and process for all samples. Extracts were analysed using a GC/MS method. The GC/MS system consisted of a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The oven temperature ranged from 70 °C to 170 °C at 5 °C min$^{-1}$, from 170 °C to 280 °C at 7 °C min$^{-1}$, and from 280 °C to 320 °C at 15 °C min$^{-1}$. The oven then remained at 320 °C for 4 min. A 30 m fused silica column (95% dimethyl siloxane, 5% phenyl polysilphenylene-siloxane, v/v) was used with helium as the carrier gas at a rate of 1 ml min$^{-1}$. A 1 µl aliquot of each sample was injected using the split mode (25:1).

The temperature of the ion source was set at 250 °C and the MS transfer line at 300 °C. Detection was achieved using electron impact ionization. Peaks were accurately annotated using both mass spectra (two specific ions) and retention times. Calibration curves were established by using samples made up of 60 pure reference compounds at levels of 1, 2, 5, 10, 20, 50, 100, 200, 500, 750, 1000, 1500, and 2000 µM. The levels of each metabolite were measured according to the corresponding calibration curve. Metabolite levels were quantified using XCalibur v2.0.7 software (Thermo Fisher Scientific Inc.).

**Gene expression quantification**

After transfer, control and treated seedlings were ground in liquid nitrogen. Total RNA was extracted using TRI Reagent® (Sigma, St. Louis, MO, USA) following the manufacturer’s protocol and quantified by Nanodrop ND-100 fluorospectrometer. Synthesis of cDNAs was carried out using an iScript™ cDNA Synthesis kit (BioRad, Hercules, CA, USA). Quantitative PCR was performed using iQ™ SYBR Green Supermix (Bio-Rad). Conditions were as follows: 95 °C for 5 min, and 40 (95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s) cycles, with a melting curve from 65 °C to 95 °C.

For each gene selected for analysis, specific primers were designed using Primer3Plus software (Table 1). Quantitative PCR was carried out in a Chromo 4® system (BioRad) real-time cycler, and fluorescence curves were analysed by Gene Expression version 1.1. software. Candidate gene expression was quantified relative to the level of expression of the housekeeping gene UBQ5 (Ramel et al., 2007).

**Statistical analysis**

Physiological and metabolic parameters were measured on at least five independent biological replicates of 20 pooled plantlets. Gene expression quantification was carried out on at least three independent biological replicates of 50 pooled plantlets. Statistical analyses were carried out with the R version 2.11.11 software. Pairwise comparisons of means used the non-parametric Mann–Whitney–Wilcoxon test. In order to test and visualize relationships between treatments and response parameters, principal component analyses (PCAs) based on the correlation matrix (Ramel et al., 2009a) were carried out using the FactoMineR package of R.

**Results**

**Characterization of residual pesticide contamination in the soil of field margins**

The level of residual pesticide contamination was quantified, in the context of a long-term ecological research area, in field margins affected by agricultural practices, and thus strongly susceptible to receive recurrent pesticide runoffs. The research area (ZAA) is located in an agriculturally intensive region in the south of the Mont-Saint-Michel Bay (Brittany, France). Agricultural landscape biodiversity and plant functional group composition in this area are significantly influenced by land-use practices and especially application rates of fertilizers and pesticides (Billeter et al., 2008; Liira et al., 2008). Plant communities of several field margins, associated with cereal crops or meadows, have been under study since 1994 in the ZAA. Five field margins, exhibiting significant

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**Table 1.** Genes selected for qRT–PCR analysis and corresponding primer sequences.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Gene product (gene abbreviation)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g05680</td>
<td>UDG-Glucosyltransferase (UGT74E2)</td>
<td>AGTCTCGAAGCCGTTAGCC</td>
<td>AATGGTCCTGGCTCCTTGG</td>
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<tr>
<td>At1g06750</td>
<td>4-Hydroxyphenylpyruvate dioxygenase (PDS1)</td>
<td>AAAACGACGCCACAGCTTCTC</td>
<td>AACGCCAGGCTCCTAAACAC</td>
</tr>
<tr>
<td>Atg07530</td>
<td>GRAS family transcription factor (SCL1f4)</td>
<td>TGGACGAGCAATTGTAAGGG</td>
<td>TGGACGATGAAACGCTCATGTC</td>
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<tr>
<td>At1g11680</td>
<td>14α-demethylase (CYP51A2)</td>
<td>AGCAATGTCGGAGTTTCTTCCC</td>
<td>AGCAATGAGGCGAACCAAG</td>
</tr>
<tr>
<td>At2g04050</td>
<td>MATE (multidrug and toxic compound extrusion) efflux protein (MATE)</td>
<td>AGCTCTGCGCAGAAGACTC</td>
<td>ATCCGGAAGGTGAAAGACTC</td>
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<tr>
<td>At2g29420</td>
<td>Glutathione S-transferase (GSTU7)</td>
<td>TGGACGATGAAACGCTCATGTC</td>
<td>AGCAATGAGGCGAACCAAG</td>
</tr>
<tr>
<td>At2g46830</td>
<td>Transcription repressor (CCA1)</td>
<td>TTAGTCATCCCAACCAAGTGG</td>
<td>ATGATAGGCGGAAAGTTGG</td>
</tr>
<tr>
<td>At3g02250</td>
<td>Ubiquitin 5 (UBQ5)</td>
<td>AGCCGTTGCTCCTAAAGAGT</td>
<td>TGGCTCCTAGTCGTTAGT</td>
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<tr>
<td>At5g10380</td>
<td>WRKY family transcription factor (WRKY75)</td>
<td>TGGCTCAGAAAGGTGGAGT</td>
<td>TGGCTCCTCCTTGAAGACG</td>
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<tr>
<td>At5g17300</td>
<td>Myb family transcription factor (RVE1)</td>
<td>AAGCAGTGGAAATGACCTG</td>
<td>TTGTTCCTGGTACGGACTC</td>
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<tr>
<td>At5g45340</td>
<td>P450 cytochrome family (CYP707A3)</td>
<td>TTACGTCGGCAGAAATCTC</td>
<td>AAGAATTGGCTCCTAGGG</td>
</tr>
</tbody>
</table>
Quantification of residual pesticide levels in soils of field margins located in the ZAA long-term ecological research area.

Data are expressed as µg g⁻¹ of fresh soil. The composition of the pesticide mixture model is given in the last line, where the data in parentheses correspond to pesticide levels expressed in µmol l⁻¹. The pesticide mixture model consisted of AMPA and hydroxyatrazine at the average concentration of all soil samples and of glyphosate and tebuconazole at the maximum concentration in single occurrences.

<table>
<thead>
<tr>
<th>Site</th>
<th>Adjacent field</th>
<th>Field code</th>
<th>Glyphosate</th>
<th>AMPA</th>
<th>2-Hydroxyatrazine</th>
<th>Tebuconazole</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>Meadow</td>
<td>11a</td>
<td>0</td>
<td>0.20</td>
<td>0.15</td>
<td>0</td>
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<tr>
<td>A</td>
<td>Meadow</td>
<td>11a</td>
<td>0</td>
<td>0.34</td>
<td>0.15</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Meadow</td>
<td>7b</td>
<td>0</td>
<td>0.42</td>
<td>0.14</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Meadow</td>
<td>7b</td>
<td>0</td>
<td>0.16</td>
<td>0.14</td>
<td>0.36</td>
</tr>
<tr>
<td>B</td>
<td>Wheat</td>
<td>11b</td>
<td>0.31</td>
<td>1</td>
<td>0.15</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Wheat</td>
<td>11b</td>
<td>0</td>
<td>0.33</td>
<td>0.15</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Maize</td>
<td>33b</td>
<td>0</td>
<td>0.48</td>
<td>0.15</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Maize</td>
<td>33b</td>
<td>0</td>
<td>0.16</td>
<td>0.14</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Maize</td>
<td>1865b</td>
<td>0</td>
<td>0.35</td>
<td>0.16</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Maize</td>
<td>1865b</td>
<td>0</td>
<td>0.28</td>
<td>0.16</td>
<td>0</td>
</tr>
<tr>
<td>Pesticide mixture model</td>
<td></td>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.15</td>
<td>0.3</td>
</tr>
<tr>
<td>C concentration</td>
<td></td>
<td></td>
<td>(1.77 µM)</td>
<td>(2.7 µM)</td>
<td>(0.76 µM)</td>
<td>(0.97 µM)</td>
</tr>
</tbody>
</table>

Effects of residual pesticide multicontamination on plant growth and development

The pesticide mixture at field margin concentration (C) significantly affected the growth and development of Arabidopsis seedlings, either by direct exposure at the start of germination (Fig. 1) or by secondary exposure of photosynthetic plants (Fig. 2). Thus, this level of residual pollution resulted in >80% inhibition of root growth (Figs 1A, 2). Dose–response analysis, using concentrations ranging from C/5 to 2C or 10C, confirmed that Arabidopsis seedlings were highly sensitive to the pesticide mixture (Figs 1, 2). C/2 concentration affected root growth of directly treated (Fig. 1) and of transferred (Fig. 2) plants. While C/5 concentration inhibited root growth of transferred plants (Fig. 2), direct exposure had no significant effects on root growth (Fig. 1). The effect on biomass (data not shown) showed the same trend. It was noteworthy, especially in the context of the edaphic localization of residual pollution, that these strong effects on root growth occurred prior to any major change in photosynthetic parameters (Fig. 1B, C), even though the archetypal effects of glyphosate on plants are related to photosynthesis inhibition (Vivancos et al., 2011). Moreover, transfer experiments (Fig. 2) showed rapid effects of pesticide exposure, with a sharp decrease of root growth within 24 h of treatment for C to 10C concentrations and within 48 h of treatment for C/5 and C/2 concentrations. C and 2C concentrations also affected chlorophyll and carotenoid levels (Fig. 1C). However, the maintenance of
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PSII efficiency at the control level indicated that photosynthesis remained active over this range of multi-pesticide mixture. It therefore appeared that low levels of residual runoff pesticide and degradation product mixtures affected specific plant physiological parameters in a dose-dependent manner.

Characterization of interactive effects between pesticides

In order to analyse the effects of pesticides in a mixture, single molecules and their various combinations were tested at their concentration in the C mixture (Figs 3, 4). The experimental set-ups for direct exposure and measurement of root growth were used to carry out a complete parallel analysis of all the combinations of AMPA (A), glyphosate (G), hydroxyatrazine (H), and tebuconazole (T) (Fig. 3).

Unlike degradation products A and H, active molecules G and T significantly affected root growth. Most combinations where G and T were present also showed significant effects. The combinations HG, GA, GT, AT, HAG, HGT, GAT, and HGAT resulted in similar root inhibition to that of G and T alone. In contrast, HA, HT, and HAT treatments gave intermediate inhibition responses that could not be ascribed to the effect of single molecules. Thus, the significant inhibition effect of HA contrasted with the absence of an effect of H or A alone (Fig. 3). For HT and HAT, plant root growth was significantly less inhibited than in the case of single T
treatment (Fig. 3). A similar trend was observed for AT treatment, which was statistically equivalent to both T and HT treatments. Moreover, this lifting of T-dependent inhibition by H or A was not observed in the case of G-dependent inhibition (Fig. 3). The main active component leading to inhibition by the HAGT mixture thus seemed to be glyphosate. Transfer experiments (Fig. 4) confirmed the effects of active molecules, G and T, relative to degradation products, with, however, a weaker effect of tebuconazole. The effects of HAG, HAT, and HAGT thus appeared to be related to the effects of active G and/or T molecules. In the transfer experiment, no lifting of T inhibition by H or A was observed during HAT treatment. The results in Figs 3 and 4 clearly showed that there was no additive effect when active molecules were combined. This comparative analysis therefore revealed that seedlings were affected not only by low levels of the herbicide glyphosate, but also by low levels of the fungicide tebuconazole. Moreover, an unexpected synergy between degradation products that were individually inactive (Fig. 3) and an unexpected antagonism between degradation products and tebuconazole (Fig. 3) were observed.

Integrative analysis reveals cryptic effects of residual pesticides and of pesticide degradation products

Integrative analyses of physiological, metabolic, and gene expression responses were carried out using comparative experiments of varying pesticide combinations (Fig. 5). The interactions between pesticides were first analysed in terms of growth, seedling biomass, chlorophyll levels, and carotenoid levels by PCA (Fig. 5A). Three major clusters were identified: (i) the control associated with AMPA and hydroxyatrazine treatments; (ii) the active pesticides glyphosate and tebuconazole; and (iii) pesticide mixtures. Responses of seedlings treated with pesticide mixtures (HAG, HAT, and HAGT) were specific and clearly distinct from the responses to single-pesticide treatments. These specificities and the absence of major physiological effects from single degradation product treatments therefore suggested the existence of, on one hand, significant interactions between active pesticides and less active degradation products, and, on the other hand, different response mechanisms to pesticide mixtures and single pesticides.

Based on the detection and quantification of metabolites (Supplementary Table S3 at JXB online) across the various treatments, metabolic profiling was characterized by PCA (Fig. 5B). Major changes of metabolic patterns were observed between control seedlings and pesticide treatments. Pesticide mixture treatments (HAG, HAT, and HAGT) induced a set of metabolic patterns that were distinct from those of single pesticide treatments (Fig. 5B), in accordance with the clustering of their physiological effects (Fig. 5A), with, however, a rather specific pattern for HAG relative to HAT and HAGT. The metabolic patterns of glyphosate, tebuconazole, and hydroxyatrazine were very similar, the latter being the closest to the control. AMPA treatment was characterized by a metabolic pattern that was clearly distinct from other patterns and by a strong impact on a wide range of metabolites, which contrasted with its absence of physiological impact (Figs 3, 4, 5A). AMPA induced major metabolic changes, revealing cryptic effects that had not been identified by morphological
Fig. 5. Principal component analysis of the physiological, metabolic, and molecular responses of *Arabidopsis thaliana* seedlings to varying combinations of hydroxyatrazine (H), AMPA (A), glyphosate (G), and tebuconazole (T). Principal component analysis was carried out on the correlation matrix of averages of physiological parameters [root growth after 14 d direct exposure (growth_d), seedling biomass after 14 d direct exposure (biomass_d), chlorophyll levels after 14 d direct exposure (chl), carotenoid levels after 14 d direct exposure (car), root growth after 72 h secondary exposure (growth_t), and seedling biomass after 72 h secondary exposure (biomass_t)], metabolite levels (B), and transcript levels (C). The metabolites and the transcripts that were analysed are described in Supplementary Table S3 online and in Table 1, respectively. Metabolites and transcript levels were quantified after 72 h secondary exposure. The position of the different pesticide treatments and the distribution of physiological, metabolic, and molecular parameters on the first plane (Dim1 and Dim2) are shown.
and physiological traits, and thus suggesting that AMPA acted on distinct and specific biochemical pathways. Finally, all treatments were found to be discriminated from control in an axis involving soluble sugars, which are related to both carbon metabolism and stress responses (Couée et al., 2006), and ascorbate, which plays an important role in abiotic stress responses (Foyer and Noctor, 2011).

Interactions between pesticides were then studied through their effects on gene expression. UGT74E2, PDS1, SCL14, MATE, GSTU7, WRKY75, and RVE1 (Table 1) were chosen according to their regulation of expression in the presence of pesticides in Arabidopsis (Ramel et al., 2007, 2012; Fode et al., 2008; Das et al., 2010). CYP51A2 was selected for its implication in Arabidopsis during development and plant growth (Kim et al., 2005) and for the involvement of a fungal homologue in azole fungicide sensitivity (Cools et al., 2006). Similarly, the CYP707A3 gene was chosen for the inhibitory effect of the plant growth retardant uniconazole on its expression (Todoroki et al., 2012). Integrative analysis of expression responses by PCA showed that these molecular markers responded to most treatments (Fig. 5C), whether as single pesticide treatment or as mixture treatment, except in the case of tebuconazole treatment, which was very similar to the control. The HAT and HAGT mixtures showed a similar expression pattern that was significantly distinct from that of the HAG mixture, as was the case for their metabolic patterns. Hydroxyatrazine was found to induce major changes in gene expression, although this degradation product did not have a large impact on morphological and physiological traits and metabolic profiles.

The major difference between these three PCA results was the distribution of single pesticide treatments, which showed a discrepancy between physiological parameter distribution and metabolic and molecular distributions. Pesticide mixtures showed some correlation of physiological effects, and important differences in molecular and metabolic profiles, thus suggesting the involvement of distinct signalling pathways. This parallel analysis of multiple physiological, metabolic, and molecular traits was therefore essential to highlight cryptic and interactive effects of residual runoff pesticides, which would not have been detected by single parameter studies. Moreover, metabolic and molecular responses emphasized the importance of signalling networks.

Metabolic and transcript variations reflect novel mechanisms and signalling pathways of pesticide impact

In order to examine the relationships between the effects of pesticides on physiological traits, metabolism, and gene expression, a PCA centred on physiological, biochemical, and molecular parameters using the same data set as in Fig. 5 was carried out (Fig. 6). This representation confirmed the fact that each pesticide and each mixture had specific effects on one or more of the traits. Physiological traits, metabolites, and transcripts were found to be significantly regulated by various treatments, whether through a decreased or increased level, as shown by the distribution of the different parameters in the PCA. Metabolites such as ascorbate, inositol, and serine, and gene expression of MATE, CYP707A3, and PDS1

**Fig. 6.** Principal component analysis of the physiological, metabolic, and molecular markers of responses of Arabidopsis thaliana seedlings to varying combinations of hydroxyatrazine (H), AMPA (A), glyphosate (G), and tebuconazole (T). Principal component analysis was carried out on the correlation matrix of averages of physiological parameters, metabolite levels, and transcript levels. The various parameters and their symbols are as described in Fig. 5. The position of physiological, metabolic, and molecular markers and the distribution of pesticide treatments on the first plane (Dim1 and Dim2) are shown. For clarity of the figure, the descriptors of physiological parameters have been omitted. (This figure is available in colour at JXB online.)
increased in response to treatments, whereas the levels of sorbitol, soluble sugars, and glutamate, and gene expression of *RVE1* and *GSTU7* decreased (Fig. 6). In contrast, other metabolic or gene pathways, such as phenylalanine, tyrosine, and *WRKY75*, were not significantly affected. Figures 7 and 8 summarize, respectively, the metabolite levels and gene expression that were most affected by pesticide treatments.

Glyphosate was the only compound with a well-described mode of action in planta. However, although it is expected to inhibit synthesis of aromatic amino acids as a result of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibition (Steinrücken and Amrhein, 1980), glyphosate did not modify the levels of tyrosine (Fig. 7) and phenylalanine (data not shown). It thus appeared that glyphosate effects in the present treatments could not be ascribed to EPSPS inhibition. Glyphosate had a negative impact on general metabolism, with a decrease of intermediate carbon (soluble sugars) and amino acid (glutamate) metabolites. It also increased stress-related compounds, such as inositol and ascorbate (Foyer and Noctor, 2011; Valluru and Van den Ende, 2011), and such as serine, which, as a cysteine precursor, is involved in glutathione metabolism (Foyer and Noctor, 2011). In contrast, its less active degradation product AMPA strongly decreased inositol, serine, glutamate, and glycine. Simultaneous depletion in glycine and serine levels agreed with the close metabolic links between serine and glycine.

The glycine/serine ratio is known to be positively correlated to the flux of C through the photorespiratory pathway (Foyer et al., 2003). The glyphosate treatment slightly reduced this ratio, whereas AMPA sharply decreased it, thus suggesting a strong effect on C photorespiratory flux. These drastic effects of AMPA on amino acid levels were not found after treatments by AMPA-containing mixtures. All of the treatments, with single pesticides or pesticide mixtures, resulted in a significant decrease of sorbitol and soluble sugars. This decrease of C compounds could be related to stress sensitivity and to a shift in carbon use. These variations in levels are therefore interesting to highlight responses to low-level pesticide contamination. In some cases, pesticide mixtures showed distinct effects relative to single pesticide treatments. It is noteworthy that the HAG mixture induced an increase in the ascorbate level, whereas each compound used separately had no effect or induced a much smaller increase. The HAGT treatment had little effect on the glutamate level despite the decreasing effects of A, G, and T alone.

Transcript levels (Fig. 8) also showed significant patterns of responses to the different pesticide treatments. The genes showing greater variation corresponded to three major functional classes, pesticide metabolism (*GSTU7* and *MATE*), metabolic stress responses (*PDS1*), and developmental stress signalling (*CYP707A3*, *RVE1*, and *WRKY75*). These three functional classes did not show coordinated expression under

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**Fig. 7.** Effects of hydroxyatrazine (H), AMPA (A), glyphosate (G), and tebuconazole (T) combinations on metabolite levels in *Arabidopsis thaliana* seedlings grown for 11 d in the absence of pesticide and transferred to fresh growth medium in the presence of varying associations of the four pesticides. In all treatments, whether single pesticide or multiple pesticide, each pesticide was at the concentration corresponding to the reference concentration (C) of the pesticide mixture described in Table 2. Values of metabolite levels (mean ±SEM) at 72 h after transfer are shown.
any of the pesticide treatments. Thus, the active molecule glyphosate induced expression of \textit{CYP707A3}, but repressed expression of \textit{GSTU7} and \textit{RVE1}. The degradation products AMPA and hydroxyatrazine and HAT and HAGT mixtures induced similar repression effects on \textit{RVE1} and \textit{GSTU7} expression. Significant effects of degradation products also affected expression of \textit{CYP707A3}, which was induced by both AMPA and hydroxyatrazine, and of \textit{MATE} and \textit{PDS1}, which were induced by hydroxyatrazine. These unexpected molecular effects of theoretically inactive components were particularly highlighted by hydroxyatrazine treatment, which resulted in greater repression or induction than other single pesticide treatments in the case of \textit{MATE}, \textit{PDS1}, and \textit{GSTU7} regulation. Finally, in contrast to its significant metabolic effects (Fig. 7), the fungicide tebuconazole showed only slight repression of \textit{WRKY75} and \textit{RVE1} transcript levels.

Pesticide mixtures showed significant effects on transcript levels, which were, at least in some cases, distinct from the effects of single molecule treatments. Indeed, field mixture HAGT treatment induced expression of \textit{MATE}, whereas the compounds taken separately induced a much smaller response or none. Moreover, HAGT repressed \textit{CYP707A3} expression, whereas the compounds taken separately induced an increase in expression. It is also noteworthy that HAG treatment had no effect on \textit{RVE1} expression, whereas compounds taken separately repressed its expression. Moreover, gene expression analysis showed that the three pesticide mixtures differed in their molecular effects, for instance in the case of \textit{PDS1}, which was greatly induced by HAG in contrast to the absence of the effect of HAT and HAGT treatments. Thus, variations of \textit{PDS1} and \textit{MATE} expression may give useful information on the impact of hydroxyatrazine and hydroxyatrazine-containing mixtures.

All of these effects, on metabolites and gene expression, strongly suggested that low levels of pesticides, whether as single molecule treatment or as mixtures, and whether as active molecules or as degradation products, interact with plant cell signalling and functioning.

**Discussion**

**Impact of persistent residual pesticide contamination**

Analysis of soil samples from field margins detected four pesticides and degradation products: glyphosate, AMPA, tebuconazole, and hydroxyatrazine. Surface water contamination in Brittany is followed monthly by the CORPEP and the RCS (Network for water control and surveillance). The first four substances most frequently quantified in 2009–2010 were AMPA, desethyl-atrazine, hydroxyatrazine, and glyphosate. The fungicide tebuconazole is frequently found in surface waters (Komárek et al., 2010). From 2007 to 2010, peak concentrations for glyphosate, AMPA, hydroxyatrazine, and tebuconazole in surface waters of Brittany varied from 2.4 to 5.42 µg l\(^{-1}\), 0.9 to 3.99 µg l\(^{-1}\), 0.04 to 0.08 µg l\(^{-1}\), and 0.15 to 11.42 µg l\(^{-1}\), respectively (CORPEP). Surface water analysis in the ZAA long-term research area, where soil sampling was done (Table 2), showed that in 2009 average concentrations of glyphosate, AMPA, and hydroxyatrazine, respectively, reached 0.11, 1.1, and 0.08 µg l\(^{-1}\). The nature of pesticides and degradation products found in soils of field margins of the ZAA (Table 2) was therefore in accordance with regional...
surface water contamination data. Bioavailable pesticides in the field fluctuate according to pesticide- and environment-related characteristics and leaching events. In order to gain deeper understanding of pollutant bioavailability, additional studies would be required concerning soluble and active fractions of pollutant mixtures in soil and experimental media.

The present results confirmed that pesticide degradation products were still found in the environment, even several years after the prohibition and disuse of parent molecules (i.e. atrazine). Experimental approaches using outdoor lysimeters have shown that atrazine and hydroxyatrazine were detected 22 years after application (Jablonowski et al., 2011). The present study demonstrates that low environmentally relevant levels of pesticides and their degradation products, whether as single molecules or as mixtures, can have a significant impact on plant functioning. The sensitivity of root growth and development to residual pesticides could have important consequences for mineral nutrition of plant communities and for seed bank dynamics. Atrazine acts on photosynthetic organisms by binding and inhibitory effects on the D1 protein of PSI and by signalling effects (Rutherford and Krieger-Liszkay, 2001; Ramel et al., 2007, 2009b). It can thus substantially affect development of A. thaliana seedlings at concentrations as low as 100 nM (Sulmon et al., 2004). Low concentrations of atrazine also affect non-photosynthetic organisms. Specific binding to the growth hormone-releasing hormone receptor at environmentally relevant concentrations (Fakhouri et al., 2010) thus links atrazine effects to endocrine disruptor activity and development of reproductive cancers in humans (Fan et al., 2007). In contrast, atrazine degradation products, and particularly hydroxyatrazine, have generally been considered to be non-phytotoxic (Shimabukuro, 1967). The present results show that this by-product did not cause any major physiological damage (Figs 3, 4). However, the results also show that gene expression was significantly affected by the presence of hydroxyatrazine (Fig. 8), thus suggesting that it could act at the molecular level on unknown targets. It has been shown, in a non-target organism such as the rat, that hydroxyatrazine delayed the onset of puberty (Laws et al., 2003), thus indicating possible endocrine disrupting-activity like atrazine. Single tebuconazole or glyphosate treatments had strong effects on root growth (Figs 3, 4). Tebuconazole is a fungicide belonging to the triazole family, which inhibits the synthesis of sterols in fungi. It has also been shown to inhibit the plant sterol 14α-demethylase enzyme from Sorghum (Lamb et al., 2001). Child et al. (1993) have shown that tebuconazole induced inhibition of stem extension and a decrease of gibberellic acid levels in oilseed rape. This inhibition was lifted by exogenous gibberellic acid, suggesting therefore that the inhibitory effects of tebuconazole could be related to interference with growth-promoting hormones. The significant effect of glyphosate (Figs 3, 4) seemed to be independent of its expected effects on aromatic amino acid levels (Vivancos et al., 2011), which remained constant upon glyphosate treatment (Fig. 7). Single AMPA treatment did not have any effect on root growth (Figs 3, 4). However, the combination of the two degradation products, hydroxyatrazine and AMPA, was found to affect root growth negatively. The effects of low environmentally relevant levels of pesticides and degradation products could not therefore be restricted to active molecules, nor ascribed to currently known mechanisms.

Integrative analysis of physiological, metabolic, and molecular responses highlights the signalling effects of pesticide environmental levels on plants

The residual pesticide mixture found in field margins had very strong effects on root growth and development, even in the absence of significant effects on photosynthesis and photosynthetic pigments (Fig. 1). These effects on root growth and development occurred in parallel with extensive metabolic changes outlined by an increase of stress metabolites (ascorbate, inositol) and a decrease of carbon metabolites (soluble sugars). However, the mechanisms of action underlying these developmental and metabolic effects remained unclear: (i) glyphosate treatment did not modify the levels of aromatic amino acids, thus suggesting that the EPSPS target of glyphosate was not affected (Vivancos et al., 2011); (ii) whereas tebuconazole had significant effects on Arabidopsis, and although triazole molecules generally act as cytochrome P450 inhibitors (Hartwig et al., 2012), no specific target of tebuconazole has been identified to date in higher plants (Child et al., 1993); (iii) whereas hydroxyatrazine had significant metabolic effects on Arabidopsis, no specific metabolic target of hydroxyatrazine has yet been identified to date in higher plants.

AMPA showed striking and specific effects on glycine levels (Fig. 7), which could result from its structural similarity to glycine (Benbrahim et al., 2008), and subsequent interactions with enzymes of glycine metabolism, such as glycine decarboxylase, serine hydroxymethyltransferase, or glutamate–glyoxylate aminotransferase (Bauwe and Kolukisaoglu, 2003; Verslues et al., 2007). However, the effects of the HGAT mixture did not result in depletion of glycine levels as occurred under conditions of AMPA treatment, thus showing the involvement of mechanisms of action other than interference with glycine metabolism enzymes.

More generally, as discussed above, the physiological, metabolic, and molecular effects of the various pesticide mixtures were distinct from the effects of single pesticide treatments, thus suggesting complex mechanisms of interaction. Despite the lack of action on well-defined metabolic targets, pesticide treatments were generally shown to act on gene expression patterns related to hormone balance and transcription, in agreement with genome-wide transcriptomic analysis of pesticide effects in plants (Ramel et al., 2007) and with previous studies on hormone–xenobiotic cross-talk (Sulmon et al., 2007; Weisman et al., 2010; Ramel et al., 2012). Thus, the CYP707A3 gene, which encodes an abscisic acid (ABA) 8′-hydroxylase that controls ABA catabolism and regulation of ABA levels (Kitahata et al., 2005), was affected by all pesticide treatments, with significant and opposite effects of hydroxyatrazine and of the HGAT mixture. The genes encoding the Myb transcription factor RVE1 (Rawat et al., 2009) and the WRKY75 transcription factor (Devaih et al., 2007) were also regulated under conditions of pesticide treatment, in particular
as a result of hydroxyatrazine, glyphosate, or tebuconazole treatments. Control of RVE1 expression and involvement of RVE1 have been related to control of auxin production (Rawat et al., 2009) and to light/carbohydrate dynamics (Usadel et al., 2008). Control of WRKY75 expression and involvement of WRKY75 have been related to root development (Devaiah et al., 2007) and H2O2 dynamics (Inzé et al., 2012). Pesticide effects on expression of regulatory genes such as CYP707A3, RVE1, or WRKY75 were therefore consistent with modifications of growth and development (Figs 3, 4), carbon metabolism (Fig. 7), and reactive oxygen species dynamics (Ramel et al., 2009b). Thus, the negative impact of a HGAT mixture on CYP707A3 and RVE1 expression may result in changes in hormonal balance (Kitahata et al., 2005; Rawat et al., 2009) leading to inhibition of primary root growth (Figs 1–4).

Such effects on hormone balance and transcription regulation genes strongly suggest pesticide interactions with mechanisms other than action on metabolic targets, thus implying that glyphosate, AMPA, tebuconazole, and hydroxyatrazine must act on unknown targets leading to gene regulation, probably through signalling processes. In the case of glyphosate, EPSPS-independent regulatory effects have been described (Vivancos et al., 2011), in agreement with the present study, but signalling targets for glyphosate interaction remain to be identified. In the case of AMPA, novel mechanisms of toxicity can be hypothesized from the present analysis of glycine levels in response to AMPA treatment (Fig. 7). However, structural analogy with glycine may also provide non-enzymatic sites of binding that remain to be identified. Tebuconazole, as a member of the triazole family, may interact through structural analogy with steroid signalling (Hartwig et al., 2012). Finally, the parent molecule of hydroxyatrazine, atrazine, has been shown to cause major gene expression reprogramming in Arabidopsis (Ramel et al., 2007, 2013) through the involvement of signalling processes (Ramel et al., 2012). Preliminary analysis indicated that hydroxyatrazine elicited hydrogen peroxide accumulation (data not shown), as was shown to be the case for atrazine (Ramel et al., 2009b). Moreover, characterization of the involvement of the AP2/ERF CRF6 transcription factor in atrazine responses (Ramel et al., 2007, 2012) demonstrates cross-talk between atrazine effects and cytokinin pathways (Cutcliffe et al., 2011), which had been previously hypothesized in studies of growth-promoting action of triazines (Nadar et al., 1975). Since these effects of triazines probably depend on the N-heterocycle (Nadar et al., 1975), it can be speculated that such cross-talk with cytokinin pathways may also be induced by hydroxyatrazine. To the authors’ knowledge, the present study presents the first evidence to date on the induction of major metabolic and molecular perturbations by pesticide degradation products. Further work is therefore required to examine these potential signalling mechanisms, which may be particularly important for the study of sublethal pesticide levels and for deciphering the interactive effects of pesticide mixtures. Characterization of such signalling and hormone cross-talk effects, which is a major field of research in animal toxicology (Frye et al., 2011), should provide novel insights into the environmental impact of runoff pesticides on plant communities.

Better insight into the signalling processes should improve mathematical models predicting the joint effect of interacting mixtures. Environmental regulations specify methods for assessing the toxicity of pollutant mixtures. The frequently used concentration addition model, or toxic units approach, adds the toxicities of individual molecules with a similar mode of action (Loewe and Muischnek, 1926), whereas the independent action model (Bliss, 1939) takes into account the toxicities of molecules that differ in their mode of action. These models have focused on ‘no-interaction’ scenarios, but the toxicity of mixtures is not always equal to the sum of single toxicities (additivity) as estimated by these approaches. There may be synergism or antagonism, as demonstrated in the present work. In order to take into account complex interactions, computational toxicology develops novel approaches taking into account molecular descriptors and computational techniques (Kim et al., 2012). However, these approaches do not readily estimate the specific involvement of all possible parameters in the resulting risk assessment. Moreover, as described by Isensee et al. (1998), variation in pollutant combination influences the availability of toxic molecules and interactions with the plant compartment. Toxicant mixtures in natural environments are complex. The quality and quantity of toxicants vary greatly and the physicochemical conditions fluctuate. The evaluation of all the necessary parameters to predict the toxicity of a residual pollutant in a natural environment is a real challenge. As highlighted in the present work, detailed knowledge of biological mechanisms, under single or multiple pollution conditions, is necessary to improve assessment and predicting tools in order to estimate environmental risks associated with residual contamination.

Supplementary data

Supplementary data are available at JXB online.

Table S1. List of the pesticides analysed in soil samples (CGI analytical platform, Ploemeur, Brittany, France).

Table S2. Hoagland medium composition

Table S3. Plant metabolites analysed by GC/MS methods

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References


Borggaard OK, Gimsing AL. 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Management Science 64, 441–456.


Jablonski ND, Schäffer A, Burauep P. 2011. Still present after all these years: persistence plus potential toxicity raise questions about the use of atrazine. Environmental Science and Pollution Research International 18, 328–331.


Laws SC, Ferrell JM, Stoker TE, Cooper RL. 2003. Pubertal development in female Wistar rats following exposure to propazine


