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Low doses of triazine xenobiotics mobilize ABA and cytokinin regulations in a stress- and low-energy-dependent manner

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ABSTRACT

The extent of residual contaminations of pesticides through drift, run-off and leaching is a potential threat to non-target plant communities. *Arabidopsis thaliana* responds to low doses of the herbicide atrazine, and of its degradation products, desethylatrazine and hydroxyatrazine, not only in the long term, but also under conditions of short-term exposure. In order to investigate underlying molecular mechanisms of low-dose responses and to decipher commonalities and specificities between different chemical treatments, parallel transcriptomic studies of the early effects of the atrazine-desethylatrazine-hydroxyatrazine chemical series were undertaken using whole-genome microarrays. All of the triazines under study produced coordinated and specific changes in gene expression. Hydroxyatrazine-responsive genes were mainly linked to root development, whereas atrazine and desethylatrazine mostly affected molecular signaling networks implicated in stress and hormone responses. Analysis of signaling-related genes, promoter sites and shared-function interaction networks highlighted the involvement of energy-, stress-, abscisic acid- and cytokinin-regulated processes, and emphasized the importance of cold-, heat- and drought-related signaling in the perception of low doses of triazines. These links between low-dose xenobiotic impacts and stress-hormone crosstalk pathways give novel insights into plant-pesticide interactions and plant-pollution interactions that are essential for toxicity evaluation in the context of environmental risk assessment.

1. Introduction

Conventional agriculture requires significant crop protection in order to reduce pest-induced yield losses. In the last fifty years, hundreds of molecules, of both natural and anthropogenic origins, have been released in environment, specifically targeting vital mechanisms in weeds, arthropods and microorganisms. Such widespread application has led to global diffusion of xenobiotic pesticides in soil and water compartments [1]. Besides desired action on pests, pesticides, such as herbicides, affect non-target organisms in agricultural areas [2]. Pesticides, and particularly herbicides, have different degrees of persistence in environmental compartments according to chemical structure and to physicochemical properties of environmental matrices [3]. Soil and water detection of herbicide-related molecules indicates that spontaneous chemical reactions, microorganism-related and plant-related processes [4] cause the liberation of degradation products, whose impacts on ecosystem balance are still poorly understood.

Atrazine (ATZ) is a selective and systemic herbicide used to control broadleaf weeds. Because of widespread use and persistence, atrazine has emerged as a pollutant of environmental concern [5]. It is currently applied in most regions of America, Asia and Oceania. Moreover, even several years after end of use, as in the European Union where it was banned in 2004, it is still detected in the environment [6]. ATZ targets D1 subunit of Photosystem II (PSII), thus leading to interruption of the photosynthetic electron transport chain, to loss of PSII efficiency, and to production of Reactive Oxygen Species (ROS) [7].

Triazines pollution in the environment involves not only ATZ itself, but also chemically-similar compounds derived from ATZ degradation. Among them, desethylatrazine (DEA) and hydroxyatrazine (HA) are frequently detected in water streams [5]. Little information is available about their effects on plants. Serra et al. [8] demonstrated that realistic environmental concentrations of HA did not affect Arabidopsis photosynthesis and growth, but induced cryptic effects modifying plant metabolism and gene expression. This apparently non-phytotoxic degradation product can therefore act on unknown molecular targets. Alberto et al. [5] recently showed that, under conditions of transient root exposure (24 h of exposure) to low doses of ATZ, DEA and HA, Arabidopsis responded to triazines with significant effects on growth

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and development in dose-dependent and differential manners. ATZ effects ranged from major impact on night-time respiration at low dose (1 µM) to multiple impacts on respiration, photosynthesis, root growth and plant growth at higher dose (10 µM). This significant impact on night-time respiration indicated that lower doses of ATZ were not innocuous for plants, even when PSII efficiency was not affected. DEA exposure resulted in significant effects on root growth, non-photochemical quenching (NPQ) and night-time respiration at high dose (10 µM). In contrast, HA caused minor and transitory physiological effects on root growth, thus highlighting the key role of chloride moiety, present in ATZ and DEA, but not in HA, for triazine toxicity. These discrepancies of effects indicate complex interactions with non-PSII targets. ATZ and DEA may affect physiological processes through respiratory and mitochondrial impacts in addition to PSII perturbation at higher levels. However, DEA also interfered with root growth independently from PSII efficiency inhibition and HA may act on root meristem activity through an alternative action mode. Such complex interactions have also been revealed in the context of sugar-induced protection against ATZ exposure [9], where ATZ did not affect photosynthesis in the short-term, but induced rapid and important gene expression variations, thus indicating underlying regulation and signaling mechanisms that remained to be characterized.

In order to determine early regulatory processes and signaling pathways involved in the action of ATZ, DEA and HA, a whole-genome transcriptomic approach was undertaken in Arabidopsis under conditions of short-term (24 h of exposure), root-level exposure to 1 µM ATZ, DEA or HA, reflecting environmental levels of diffuse or transient pollution. This genome-wide approach revealed specific signatures and common responses. Detailed analysis of these patterns highlighted potential disruption of energy, carbon and nitrogen homeostasis, and potential activation of cell-wall and defense processes. The significant proportion of transcription- and signaling-related genes in the sets of differentially-expressed genes provide novel insights into the primary signaling components that are early affected by triazines in the course of plant-xenobiotic interactions.

2. Material and methods

2.1. Plant material and growth conditions

Seedlings of *Arabidopsis thaliana* (ecotype Columbia, Col0) were cultivated after seed sterilization as described in Alberto et al. [5]. RNA extractions for microarray and qRT-PCR experiments were performed on thirteen-d-old plantlets transferred at 1.04 developmental growth stage [10] on control or to triazine [1 µM ATZ, DEA or HA (Sigma, St. Louis, MO, USA)] 0.8% agar-Hoagland media during 24 h. Each treatment was repeated in order to obtain independent biological and experimental replicates. In each replicates, 60 plantlets consisting in the pool of 3 distinct sets of 10–15 individual plants treated as described above and corresponding to a given treatment were harvested, frozen in liquid nitrogen and extracted for RNA.

2.2. Transcriptome profiling microarray analysis

Microarray analysis was carried out at the Institut of Plant Sciences Paris-Saclay (IPS2, Orsay, France), using the CATMAv7 [11] based on AGILENT technology on the Transcriptomic Plateform POPS (transcriptOmic Platform of iPS2). The CATMAv7 design of *Arabidopsis thaliana* genome have been made with gene annotations include in FLAGdb++, an integrative database around plant genome (http://urgv.evry.inra.fr/FLAGdb). The single high density CATMAv7 microarray slide contains four chambers, each containing 149 916 primers. Each 60 bp primer is triplicate in each chamber for robust analysis and in both strand. As part of all probes, 35 754 in triplicate correspond to gene TAIRv8 (among which 476 probes corresponding to mitochondrial and chloroplast genes) + 1289 probes corresponding to EUGENE software predictions + 658 probes for miRNA/MIR, and finally 240 controls. For each condition, RNA extractions for microarray analysis were performed on two independent biological and experimental replicates. Total RNA for each replicate was extracted from 60 thirteen d-old plantlets transferred at 1.04 developmental growth stages [10] on four different treatment media during 24 h as indicated above using QIAGEN RNeasy Plant Mini Kit, according to the supplier's instructions. For each comparison, two technical replicates with fluorochrome reversal were performed for each biological replicate (i.e. four hybridizations per comparison). The labeling of cRNAs with Cy3-dUTP or Cy5-dUTP was performed as described in Two-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling manual (© Agilent Technologies, Inc.). The hybridization and washing were performed according to Agilent Microarray Hybridization Chamber User Guide instructions (© Agilent Technologies, Inc.). Two micron scanning was performed with InnoScan900 scanner (Innopsys^R, Carbonne, FRANCE) and raw data were extracted using Mapix^R software (Innopsys^R, Carbonne, FRANCE). ATZ, DEA and HA conditions were compared to the control.

2.3. Statistical analysis of microarray data

Experiments were designed with the statistics group of the IPS2. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). For each array, a global intensity-dependent normalization using the loess procedure [12] was performed to correct the dye bias. The differential analysis is based on the log-ratios averaging over the duplicate probes and over the technical replicates. Hence the numbers of available data for each gene equals the number of biological replicates and are used to calculate the moderated t-test [13]. Under, the null hypothesis, no evidence that the specific variances vary between probes is highlighted by Limma and consequently the moderated t-statistic is assumed to follow a standard normal distribution.

To control the false discovery rate, adjusted p-values found using the optimized False Discovery Rate (FDR) approach of Storey and Tibshirani [14] are calculated. We considered as being differentially expressed the probes with an adjusted p-value ≤ 0.05. Analysis was done with the R software [15]. The function SqueezeVar of the library Limma has been used to smooth the specific variances by computing empirical Bayes posterior means. The library kerfdr has been used to calculate the adjusted p-values.

2.4. Analysis of microarray data

Hierarchical clustering was performed using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster) and Java Tree View (http://jtreeview.sourceforge.net). GO annotation retrieval and GO Slim Classification developed at TAIR (http://www.arabidopsis.org/tools/bulk/go/index.jsp) were used to retrieve GO and GO Slim terms associated with differentially expressed genes (DEGs). Over-represented and under-represented functional categories were determined by Classification SuperViewer (http://bar.utoronto.ca/) with normalized class score option. Putative shared-function and protein-protein interaction networks were determined by mapping DEGs onto STRING v.10 global Arabidopsis networks [16].

Promoter search for cis-elements was undertaken with Arabidopsis promoter motif search programs using 2 kb upstream regions from translation start sites (http://stan.cropsci.uiuc.edu/cgi-bin/elefinder/compare.cgi). Overrepresentation of octameric sequences derived from promoter consensus sequence (ACT)(AG)(GT)AT(ACT)(CT)(ACGT) was estimated as described by Brenner and Schmülling [17]. Motifs in promoters were counted using Promomer (http://bbc.botany.utoronto.ca/ntools/cgi-bin/BAR_Promomer.cgi) and significantly enriched motifs for DEG promoters were calculated against a control set of least likely CK-induced genes [17].