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Transcriptomic response of *Arabidopsis thaliana* roots to naproxen and praziquantel

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ABSTRACT

Exposition to pharmaceutical compounds released to the environment is considered as a potential risk for various organisms. We exposed *Arabidopsis thaliana* plants to naproxen (NAP) and praziquantel (PZQ) in 5 μM concentration for 2 days and recorded transcriptomic response in their roots with the aim to estimate ecotoxicity and to identify gene candidates potentially involved in metabolism of both compounds. Nonsteroidal anti-inflammatory drug NAP up-regulated 105 and down-regulated 29 genes (p-value ≤ 0.1, fold change ≥ 2), while anthelmintic PZQ up-regulated 389 and down-regulated 353 genes with more rigorous p-value ≤ 0.001 (fold change ≥ 2). High number of up-regulated genes coding for heat shock proteins and other genes involved in response to biotic and abiotic stresses as well as down-regulation of genes involved in processes such as cell proliferation, transcription and water transport indicates serious negative effect of PZQ. NAP up-regulated mostly genes involved in various biological processes and signal transduction and down-regulated mainly genes involved in signal transduction and electron transport or energy pathways. Further, two cytochrome P450s (demethylation) and one methyltransferase (methylation of carboxyl group) were identified as candidates for phase I and several glutathione- and glycosyltransferases (conjugation) for phase II of NAP metabolism. Cytochrome P450s, glutathione and glycosyltransferases seem to play role also in metabolism of PZQ. Up-regulation of several ABC and MATE transporters by NAP and PZQ indicated their role in transport of both compounds.

1. Introduction

Environmental impact of pharmaceuticals has been intensively studied during the last years. The reason is potential health and environmental risks caused by pharmaceuticals and their metabolites released into the soil and water. As human and veterinary pharmaceuticals are biologically active compounds and their use is still increasing (it raises a question “why”), there is possibility that they can negatively affect non-target organisms in various environments (Arnold et al., 2013). Non-steroidal anti-inflammatory drugs (NSAIDs) and anti-parasitic drugs belong to the compounds most used in human and veterinary medicine, respectively (Bártíková et al., 2016; Bessone, 2010). In this study we focused to naproxen and praziquantel. Naproxen ((+)−6-methoxy-α-methyl-2-naphthalene acetic acid; NAP) has been widely used as NSAID to relieve from pain, fever, inflammation, rheumatoid arthritis, psoriatic arthritis, and gout (Hutt and Caldwell, 1983). NAP together with ibuprofen, diclofenac, ketoprofen and acetaminophen were the most frequently detected NSAIDs in waste water treatment plant effluents in various global locations (Verlicchi et al., 2012). Maximum concentrations of NAP in the surface waters were 1423.8 ng/L in the Czech Republic rivers (Marsik et al., 2017a), 41 ng/L in Tehran rivers (Eslami et al., 2015), 40 ng/L in Shanghai Huangpu River system (Wen et al., 2014) and 6.4 ng/L in Finland River Vantaa (Lindholm-Lehto et al., 2016). LC_{50} and NOEC (no observed effect concentration) value for carp *(Cyprinus carpio)* determined for NAP were 269 and 25 mg/mL, respectively, EC_{50} and NOEC for crustacean *Daphnia magna* 47 and 1 mg/mL, respectively, and IC_{50} and NOEC for bacteria *Vibrio fischeri* 20 and 2 mg/mL, respectively. Acute toxicity of NAP was therefore relatively low (Gheorghe et al., 2016). However, NAP caused oxidative stress and DNA damage in *Daphnia magna* at concentration of 0.017 mg/L (Gómez-Oliván et al., 2014). Melvin et al. (2014) observed weak negative effects in striped marsh frog

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(*Limnodynastes peronii*) tadpole development following chronic exposures to NAP at concentration of 10 and 100 μg/L. NAP, NAP sodium salt and NAP phototransformation products exerted chronic toxicity with 50% population growth/reproduction inhibition values towards algae *Pseudokirchneriella subcapitata* ranging from 1.9 to 39.3 mg/L, rotifer *Brachionus calyciflorus* ranging from 0.25 to 0.79 mg/L, and crustaceans *Ceriodaphnia dubia* ranging from 0.06 to 1.06 mg/L (Isidori et al., 2005). Above cited data indicate that NAP represents relatively low environmental risk with possible chronic toxicity in concentrations similar to those in waste water treatment plant effluents. Praziquantel (2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro- 4H-pyrazino[2,1-*a*]-isoquinolin-4-one; PZQ) has been used as a broad-spectrum anthelmintic in veterinary and human medicine. PZQ is the drug of choice for treating schistosomiasis in humans (Dayan, 2003; Watson, 2009). PZQ has been also applied in aquaria to treat fish ectoparasites (Thomas et al., 2016). Information about its occurrence in the environment is scarce. Periša and Babic (2014) found PZQ in waste water treatment plant for veterinary industry in the concentration of 15.02 μg/L. On the other hand, PZQ was not detected during the survey of Spanish surface waters (Zrnčić et al., 2014). Also data about ecotoxicity of PZQ are limited. LC_{50} (96 h) value for African catfish (*Clarias gariepinus*) was 53.52 mg/L and sublethal concentrations (5.35 and 10.7 mg/L) induced micronucleus formation and alterations of hematological and biochemical parameters (Nwani et al., 2014). PZQ showed very low toxicity towards dung beetle *Aphodius constans* (LC_{50} > 1000 mg of active substance per kilogram of dung dry weight) in comparison with other antiparasitic drugs such as ivermectin (LC_{50} = 0.88–0.98 mg of active substance/kg dung dw) or dicyclanil (LC_{50} = 1.5–6.0 mg of active substance/kg dung dw; Hempel et al., 2006). Nevertheless, in recent studies metabolism of pharmaceuticals including NAP and PZQ in plants was investigated. The rationale was possible risks caused by accumulation of pharmaceuticals and their metabolites in crop plants cultivated on soils irrigated with wastewater or fertilized with biosolids (Wu et al., 2016) as well as the potential use of plants for decontamination (phytoremediation) of polluted waters (Marsik et al., 2017b). Accumulation of pharmaceutical products in crops and their possible impact on the human health were recently reviewed by Wu et al. (2015) and by Miller et al. (2016). The issues associated with phytoremediation of pharmaceutical products were reviewed by Zhang et al. (2014).

In this study, we exposed *Arabidopsis thaliana* plants to NAP and PZQ and recorded transcriptomic response in their roots using whole genome microarrays. The aim has been to elucidate how the plants are affected by these two different class pharmaceuticals at molecular level. Moreover, we pin-pointed genes potentially involved in NAP and PZQ metabolism and detoxification.

2. Materials and methods

2.1. Treatment of plants with NAP and PZQ

Arabidopsis thaliana (wild type, cv. Columbia 0) plants were cultivated in hydroponic condition using Araponics system (Araponics SA, Belgium). Containers contained 1.85 L of 25% Hoagland's solution (Hoagland, 1920) with pH adjusted to 6.2–6.3. Medium was changed every week with the fresh one and aerated every three h for 15 min. Plants were cultivated at 21 °C and 8/16 light/dark period at light intensity of 130 μmol/m^2/s in a growth cabinet (MLR-350, Sanyo Electric Co., Japan). Four week old plants were exposed to NAP applied to the cultivation medium as naproxen sodium salt (purity 100%; Sigma-Aldrich, MO, USA) dissolved in 185 μL of distilled water and to PZQ (purity > 98.5%, Glentham Life Sciences Ltd, UK) dissolved in 185 μL of ethanol (final concentration of ethanol in the hydroponic medium was 0.01%) to obtain a final 5 μM concentration. After two-day exposure, the roots were harvested, washed in deionized water, frozen in the liquid nitrogen and stored in − 80 °C. For the toxicity test four week old plants were exposed to NAP applied to the cultivation medium as naproxen sodium salt dissolved in 2 mL of distilled water to obtain final 5, 50, and 500 μM concentrations and to PZQ dissolved in 2 mL of DMSO. Untreated plants and plants exposed to 2 mL of DMSO, respectively, were used as controls. After one-week exposure fresh weighed of rosettes and roots was determined. The statistical significance of the differences among treatments was evaluated using one-way ANOVA test followed by post-hoc Tukey's test at level $p < 0.01$ (n = from 7 to 9).

2.2. Microarray analyses

RNA was extracted from the roots exposed to NAP, PZQ and control untreated plants using Plant RNA Isolation Mini Kit (Agilent Technologies, CA, USA). Isolated RNA was labeled with Low Input Quick-Amp Labeling Kit (Agilent Technologies) by Cyanine 3 and Cyanine 5 using a dye swap design to avoid dye-based bias. Labeled cRNA was purified using RNeasy Plant Mini Kit (Qiagen, Germany), fragmented and hybridized on microarray slides (Arabidopsis V4 Gene Expression Microarray; Agilent Technologies) according to manufacturer´s instructions. After hybridization the slides were washed in GE Wash Buffers (Agilent Technologies), acetonitrile, and Stabilization and Drying Solution (Agilent Technologies). The microarrays were scanned using a GenePix 4000B scanner controlled by GenePix Pro Microarray Analysis Software (Molecular Devices, CA, USA). The experiments were repeated four times with root cRNA prepared independently from individual plants. The data acquired from the scanner were processed in an R scripting environment using the software package LIMMA according to Smyth and Speed (2003), Smyth (2004), and Smyth et al. (2005). The LOESS normalization method was used to balance the mean fluorescence intensities between the green and red channels in the frame of single arrays, and the Aquantile method was used to normalize the signals among the arrays. The background intensity was not subtracted from the overall spot intensities. The statistical analyses were performed without spots with zero weights. The false discovery rate (FDR) method was used for statistical evaluation. Genes with a 2 > fold change in expression (p-value < 0.1 in the case of NAP and p-value < 0.001 in the case of PZQ) were selected. Functional classification of the up-regulated and down-regulated transcripts was performed using the Classification SuperViewer (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi; Provart and Zhu, 2003).

2.3. Quantitative real-time PCR analysis

The transcription levels obtained by microarray analyses were verified using quantitative reverse-transcription real-time PCR (qRT-PCR). The same RNA samples isolated for microarray analyses were treated with rDNase from NucleoSpin RNA Plant kit in accordance with the manual (Machery-Nagel, Germany). cDNA was synthetized using M-MLV Reverse Transcriptase (RNase H Minus – Point Mutant, Promega, WI, USA), $dT_{23}dV$ primers and the Protector RNase Inhibitor (Roche Applied Science, Germany). cDNA (20 × diluted) was added in volume of 2.5 μL into the mix of the LightCycler 480 DNA SYBR Green I Master (Roche Applied Science, Germany) and 500 nM gene-specific primers to achieve the final volume 10 μL. The qPCR was performed with the Light Cycler 480 (Roche Applied Science, Germany). PCR program was set on initial denaturation (5 min, 95 °C) followed by 45 cycles of primer denaturation (10 s, 95 °C), annealing (10 s, 60 °C) and elongation (10 s, 72 °C). The relative content of RNA was calculated (Hellemans et al., 2007) with respect to the reaction efficiency of each primer pair. Genes *UBQ10* and AT1G07940 were used for normalization. The primer sequences are stated in Supplementary Table 1.