

Detoxification of polycyclic aromatic hydrocarbons (PAHs) in *Arabidopsis thaliana* involves a putative flavonol synthase

Juan C. Hernández-Vega¹, Brian Cady¹, Gilbert Kayanja¹, Anthony Mauriello¹, Natalie Cervantes¹, Andrea Gillespie¹, Lisa Lavia¹, Joshua Trujillo¹, Merianne Alkio², Adán Colón-Carmona^{1*}

1. Department of Biology, University of Massachusetts Boston, 100 Morrissey Blvd., Boston, MA 02125, USA

2. Calvinstr. 25, D-24114 Kiel, Germany

* Corresponding author. Tel.:617-287-6680; fax: +1 617 287 6650.

E-mail address: adan.colon-carmona@umb.edu (A. Colón-Carmona).

DETOXIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN ARABIDOPSIS THALIANA INVOLVES A PUTATIVE FLAVONOL SYNTHASE

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) pollution has a negative impact on the environment. PAHs are a group of over 100 toxic organic compounds that consist of two or more benzene rings fused together and are hydrophobic, highly stable in the environment, carcinogenic, cytotoxic, teratogenic and mutagenic [1-6]. The US Environmental Protection Agency has identified 16 prominent PAHs based in their concentrations in the environment and harmful effects [7]. Thus the presence of these and other contaminants in the environment has stimulated the development of biological strategies to remediate contaminated soils [8].

Phytoremediation is a cost effective and innovative technology that involves the use of plants to remove or degrade pollutants in the environment [9-14]. It is known that plants may translocate organic pollutants from contaminated soil or water into root and shoot tissue where PAHs can be transformed by its own enzymatic machinery [15-22]. The three-phase detoxification pathway to metabolize contaminants in plants is divided into: transformation (phase I), conjugation (phase II) and sequestration (phase III) [23- 25]. Phase I transformation reactions include hydrolysis, reduction and/or oxidation of the xenobiotic compounds. These reactions turn possible toxins into less hydrophobic compounds and increase their reactivity. Phase II involves conjugation to glutathione, amino acids, or sugars, thereby producing hydrophilic polar compounds. Phase III involves the sequestration of soluble conjugates into an organelle such as the vacuole and/or the cell wall [26-31]. Thus, to effectively use plants in remediating PAH-contaminated environments, it is critical to understand the genetics and enzymatic machinery behind PAH detoxification.

Using *Arabidopsis thaliana* as a model, the PAH-induced stress response has been described [32-34]. For example, phenanthrene, a three ringed PAH, is detrimental to the growth of plants. It has significant effects on root length, leaf number and overall plant size. The photosynthetic capacity is impacted [35, 36]. Moreover, plants exposed to PAHs generate excess levels of reactive oxygen species (ROS) that damage cell structures and causes cell death [32, 33, 37-43]. A global genome transcriptional analysis of 21 day-old plants continuously grown on 0.25 mM phenanthrene showed an up-regulation of genes involved in abiotic stresses, a pathogen mediated defense responses and detoxification systems [34]. Important for this study, plants grown under phenanthrene exposure have increased messenger RNA levels of the following dioxygenase (DOX) genes: *At3g55970* (a putative oxidoreductase), *At5g05600* (a putative flavonol synthase), *At3g51240* (a putative flavanone 3-hydroxylase), *At4g22870* (a putative leucoanthocyanidin dioxygenase), *At1g14130* (a putative 2-oxoglutarate-dependent dioxygenase), and *At5g64250* (a putative 2-nitropropane dioxygenase) [34]. Bacterial DOXs, such as naphthalene dioxygenase from *Pseudomonas putida*, are known for their potential to modify PAHs by oxidizing their structure, thus increasing their reactivity [13, 22, 44- 46]. Some plant DOXs are non-heme iron-containing enzymes playing important roles in biosynthetic pathways of hormones and secondary metabolites such as flavonoids [47]. However, the role of plant DOXs in PAH detoxification is unclear.

Herein, we characterized the role of *Arabidopsis* AT5G05600 in the catabolism of PAHs. We hypothesize that plants are able to transform and degrade aromatic xenobiotics with enzymes involved in the synthesis of aromatic secondary metabolites such as flavonoids [48]. We took a combined molecular, genetic and biochemical approach to study the role of AT5G05600, a

putative dioxygenase from the flavonol synthase family, in chemically modifying phenanthrene (Figure 1). Our results indicate that AT5G05600 is necessary in responding to phenanthrene exposure and that there is an accumulation of toxic intermediates during phenanthrene biotransformation resulting from AT5G05600 function.

2. Materials and Methods

2.1 Plants and growth conditions

Arabidopsis thaliana seeds ecotype Columbia (Col-0) and Landsberg *erecta* (Ler) were obtained from the Arabidopsis Biological Resource Center (ABRC), while the loss-of-function plant mutant lines SM_3_32880, GT_5_94951 and GT_5_96064 were obtained from the ABRC and the European Arabidopsis Stock Centre (NASC). The seeds of the transgenic line cMyc-HDA6 were kindly provided by C. Pikaard [49]. The seeds were surface sterilized before being stratified at 4°C for 2 days. Then, they were grown in square petri dishes with half-strength Murashige and Skoog (MS) medium supplemented with 1.5% w/v sucrose, 0.9% w/v phytoagar, pH 5.7, and containing 0.1 mM or 0.25 mM phenanthrene. Plates were positioned vertically in a 19 °C incubator under long-day conditions with a 16/8 h photoperiod at approximately 130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. After 14 days, the plates were removed, scanned and the length of the main root was determined using NIH ImageJ software.

2.2 Histochemistry

The β -glucuronidase (GUS) staining was performed as described by Jefferson et al. [50]. Accumulation of H_2O_2 in the leaves was determined by diaminobenzidine (DAB) staining following the methods described by Thordal-Christensen et al. [51].

2.3 *In vitro* Assays

The full-length *At5g05600* cDNA from *Arabidopsis thaliana* (clone G12898) was obtained from the ABRC. Using the LR reaction (Gateway cloning technology, Invitrogen™), the cDNA was sub-cloned into the T7 promoter expression vector pDEST17 to obtain N-terminally fused 6xHis-tagged proteins. The culture of *Escherichia coli* BL-21 transformed with the expression vector was grown at 37°C to mid-log phase and induced with 0.2 mM isopropylthio- β -galactoside (IPTG). After 20 h, the cells were spun at 4000 rpm for 30 min at 4°C. The bacterial pellets were sonicated and the lysates were centrifuged at 5,000 rpm for 20 min at 4°C. Afterwards the proteins in the supernatant were purified under native conditions (The QIAexpressionist™). To obtain wash and elution fractions, the columns were treated with washing buffer and followed by elution buffer, according to manufacturer's specifications. Samples were taken from each fraction and analyzed on a SDS-PAGE.

The *in vitro* assays were based on the method described by Britsch and Grisebach [52] with few modifications. Assays were performed with 4 μ g or 6 μ g of the purified enzyme, 0.2 mM phenanthrene, 10 mM ketoglutaric acid (disodium salt), 10 mM ascorbic acid, 0.25 mM ferrous sulfate and 0.6 mg catalase. The tubes were incubated at 30° C in the dark for 2 hrs under aerobic conditions. Assays were terminated by adding 500 μ l hexane and vortexing for 1 min. The organic phase was extracted, placed in a new tube and dried overnight. Samples were resuspended in hexane and analyzed with a scanning fluorometer and High Performance Liquid Chromatography (HPLC). The chromatographic analysis was performed using a column Agilent Zorbax SB-C18 (Agilent technologies) with conditions similar to Wu et al. [53].

2.4 Generation of the *At5g05600* over-expression transgenic plant lines

The full-length cDNA of *At5g05600* (G12898) was transferred into pEarly203 following standard Gateway® procedures. The resulting over-expression *35S::Myc-AT5G05600* construct was introduced into *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis thaliana* (Col-0) plants were transformed by the floral dip method [54]. Transformants and homozygous lines were selected using 250 mg/L glufosinate-ammonium (Basta).

2.5 Semi-qualitative expression analysis

Total RNA extraction from leaf tissue isolated from the over-expression lines was performed using Qiagen RNeasy miniprep kit (Qiagen). Samples were then treated with DNase I and the single strand cDNAs were synthesized with SuperScript III kit (Invitrogen™). The cDNAs were amplified with the following primers: primer A 5'-GAACAGAACTGATCTCTGAAGAAG-3', primer B 5'-AGAAGGGTTGTCTGATGACG-3', and primer C 5'-TGGGATTTGCCTTGTGGACC-3'. RT-PCR analysis of actin (*AT2G37620*) mRNA levels using primers (5'-CACATACATAGCAGGGGCATTG-3', and 5'-GATGGTGAAGACATTCAACCTCTTG-3') was used as a control.

For protein analysis, 100 mg of plant leaf tissue from over-expressing plant lines were ground with a mortar and pestle in 500 µl of Laemmli buffer. Extracted proteins were separated in SDS-PAGE gel for two hours at 85 V. Proteins were transferred into a PVDF membrane overnight in a constant current of 35V at 4°C. Then the membrane was incubated overnight with a 1:1000 dilution of mouse anti-Myc antibodies (Covance) and later incubated for 2 hr with HRP-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, Inc.). Finally, this was processed with SuperSignal® West Dura, a luminol-based enhanced chemiluminescence

(ECL) horseradish peroxidase (HRP) substrate, to determine the presence of the recombinant protein before being analyzed.

2.6 Sequence alignments

The Arabidopsis Information Resource (TAIR) and the National Center for Biotechnology Information (NCBI) were used as databases to retrieve protein sequences. The CLUSTALW2 software was used for the analysis of multiple protein sequences. CDD [55] and ExPASy-PROSITE were used for protein domains analysis.

3. Results

3.1 Phenanthrene induces *At5g05600* expression

To study the tissue-specific expression of the *At5g05600* gene under PAH exposure, plants of the gene trap line GT_5_96064 were grown in media containing 0 mM, 0.1 mM and 0.25 mM phenanthrene for 14 days. This plant line contains a transposon with the β -glucuronidase (GUS) reporter gene [56] in the second exon of *At5g05600*, leading to no detectable full-length *At5g05600* mRNA (data not shown). Therefore, the insertion mutation is a loss-of-function for *At5g05600* and has GUS expression driven by the *At5g05600* promoter, providing *At5g05600* expression information. Transgenic plants with the GUS reporter gene growing on phenanthrene-containing media showed an increase in gene expression in the cotyledons, vascular tissues and new leaves; whereas plants not exposed to phenanthrene showed low levels of *At5g05600* expression (Figure 2). Thus, these data show that *At5g05600* is PAH inducible and confirms previous microarray studies [34].

3.2 Loss-of-function mutant lines are less sensitive to phenanthrene than control plants

To characterize the *in vivo* role of *At5g05600* under PAH-induced stress, loss-of-function mutant lines SM_3_32880, GT_5_94951 and GT_5_96064 were identified, and then their response to phenanthrene was evaluated. The Col SM_3_32880 line contains a Spm transposon in the first exon of *At5g05600*, whereas GT_5_94951 and GT_5_96064 in the *Ler* background have Ds insertions in the second exon of the gene.

Root lengths were used to quantify phenotypic changes under different phenanthrene concentrations: 0 mM, 0.1 mM and 0.25 mM. **To control for experimental variability, control wild type and experimental plant lines were grown side-by-side under identical conditions and as reported by Alkio et al [32].** The root lengths of 14 day-old plants grown in phenanthrene-containing media showed that the loss-of-function mutant lines were less sensitive to phenanthrene compared to control plants, Col and *Ler* (Figure 3). For instance, while *Ler* showed a root growth reduction of more than 30% in media containing 0.1 mM phenanthrene, the loss-of-function mutant lines GT_5_94951 and GT_5_96064 did not have a significant difference (Figure 3B, C). A less sensitive phenotype was also observed in a higher concentration of 0.25 mM phenanthrene. SM_3_32880 exhibited a reduction of 27%, whereas control Col line exhibited a reduction of 71% (Figure 3A). The less sensitive phenotype of these loss-of-function mutant lines compared to control genetic lines shows a possible involvement of *At5g05600* in the catabolism of PAHs *in vivo*.

3.3 Over-expressing plant lines are hypersensitive to phenanthrene

To further test the hypothesis that *At5g05600* has a role in PAH detoxification, this gene was over-expressed in *Arabidopsis*. Of the fifteen different transgenic lines analyzed, six

independent transgenic lines with a 3:1 segregation ratio were taken to homozygosity and used in physiological experiments. Of those, data from over-expressing lines 2B1Bp1, 3D1Ap1 and 3D1Ap2 are presented in this study. In these lines, the cDNA for *At5g05600* is under the control of the cauliflower mosaic virus (CaMV) 35S promoter, which drives transgene expression in a constitutive manner [57, 58]. The construct used also added a Myc-tag on the N-terminus of the protein. Expression analysis showed the presence of the recombinant mRNA in the over-expressing lines and absence of this message in wild type plants. Data of the line 3D1Ap1 is shown (Supplementary material A, B). The transcript levels in the over-expressing lines were significantly increased compared to the levels of the untransformed Col control (Supplementary material A, C). Importantly, the levels of the Myc-tagged recombinant protein were evaluated by SDS-PAGE and western blot, indicating the transgenic lines expressed a protein of the expected 45.5 KDa size (Supplementary material E); whereas, the recombinant protein is not present in the control plants.

To evaluate PAH tolerance and the ability to overcome PAH-induced stress, the transgenic lines over-expressing *At5g05600* were grown for 14 days in 0.5 MS media containing 0, 0.1 and 0.25 mM phenanthrene. Whereas under control conditions, no difference in root growth was observed between the wild type and over-expressing lines, the transgenic lines growing in media with phenanthrene showed a hypersensitive behavior when compared to controls (Figure 4). A dramatic difference in the response was observed with the transgenic line 3D1Ap1 (Figure 4A). This transgenic line was severely affected growing 34% and 53% less than Col on 0.1 and 0.25 mM phenanthrene, respectively. Moreover the root lengths of the over-expressing lines 3D1Ap2 and 2B1Bp1 were also significantly shorter than in wild-type plants under phenanthrene

exposure (Figure 4B, C). These data suggest that the over-expression of *At5g05600* creates PAH hypersensitivity in plants. The observed hypersensitivity in the over-expressing lines further suggests that *At5g05600* may be involved in the catabolism of phenanthrene, possibly generating the accumulation of harmful metabolites that may inhibit growth in the plant.

3.4 Leaf area analysis also shows a hypersensitive response to phenanthrene in over-expressing lines

Because root and shoot tissue respond differently to PAH exposure [33] and to determine the effects of phenanthrene on shoots of *At5g05600* over-expressing plants, the foliage of 3 week-old plants growing in round petri dishes was scanned and analyzed. The *At5g05600* over-expressing plants were found to be significantly smaller than wild type plants under phenanthrene exposure (Table 1). When exposed to 0.1 mM phenanthrene, the over-expressing lines 3D1Ap1, 3D1Ap2 and 2B1Bp1 exhibited a reduction in leaf area of approximately 25%, 20% and 14%, respectively, compared to Col control. The foliage area of the loss-of-function mutant line SM_3_32880 showed a reduction of 10% more compared to Col on 0.1 mM phenanthrene; however, it did not show a significant difference on 0.25mM phenanthrene. The loss-of-function mutant lines GT_5_94951 and GT_5_96064 did not show a significant difference compared to *Ler*, the genetic background of the mutant lines. The PAH growth response of the shoot versus root tissue suggests variability in the response between natural accessions of *Arabidopsis* and that root cells are likely more sensitive to intracellular levels of phenanthrene.

3.5 *At5g05600* over-expressing lines accumulate high levels of H_2O_2 after phenanthrene treatment

Xenobiotics cause oxidative stress in animals and plants. Wild type plants under PAH exposure accumulate ROS [32, 33, 37-43]. In this study, the production of H_2O_2 was monitored with 3, 3'-diaminobenzidine (DAB) staining in plants grown for 14 days in media with phenanthrene. DAB reacts with H_2O_2 producing a brown precipitate [51]. After treatment with 0, 0.1, or 0.25 mM phenanthrene, the loss-of-function mutant lines experienced significantly less oxidative stress versus control plants under PAH stress (Figure 5). For example, SM_3_32880 did not show any H_2O_2 accumulation during the different treatments. The control plants showed H_2O_2 accumulation, as was seen for the formation of dark brown spots, depending on the PAH concentration, similar to what has been previously reported [32, 33]. On the other hand, intense spotted staining with DAB was observed in leaves of the over-expressing lines 3D1Ap1, 3D1Ap2, and 2B1Bp1, when exposed to both 0.1mM and 0.25 mM phenanthrene (Figure 6). The higher accumulation of H_2O_2 in over-expressing lines exposed to phenanthrene suggests more oxidative damage due to PAH exposure.

3.6 Fluorescent emission and HPLC analysis of *in vitro* biochemical assays with recombinant AT5G05600 protein suggest phenanthrene chemical modifications

To determine if the AT5G05600 protein was able to chemically modify phenanthrene, the 6xhistidine (6xHis)-tagged recombinant protein was purified from crude bacterial lysates under non-denaturing conditions by nickel affinity chromatography. The collected fractions were analyzed by SDS-PAGE (Figure 7). Analysis of the elution fractions 2 to 4 showed the presence of a band of the expected size (46 KDa) for the 6xHis-tagged recombinant protein. To verify the activity of tagged AT5G05600 with relation to phenanthrene, *in vitro* assays were performed with the purified enzyme. The emission spectrum of a compound can provide qualitative and quantitative information [59, 60]. This is especially true of PAHs when exposed to UV light. For

instance when excited at 337 nm, phenanthrene produces a unique spectral emission signature with peaks at 347, 356, 364, and 374 nm [61]. Changes in the emission spectrum of a compound are associated with structural changes. Incubation of 0.2 mM phenanthrene with 4 µg or 6 µg of purified recombinant AT5G05600 revealed a reduction in the area under the curve of its fluorescence spectra (Figure 8). Using the 364 peak as an arbitrary standard peak, 0.2 mM phenanthrene produced a major peak with a measurement of approximately 8×10^4 counts per second (cps) (Figure 8A). Incubation of 0.2 mM phenanthrene with 4 µg of recombinant protein exhibited a substantial reduction to 6×10^4 cps (Figure 8B). Furthermore, incubation of 0.2 mM phenanthrene with 6 µg of the enzyme had a larger reduction to 4.5×10^4 cps (Figure 8C). Thus, these results showed a reduction of the phenanthrene amount in solution concomitant with increases in the enzyme concentration, suggesting that AT5G05600 is able to chemically modify phenanthrene *in vitro*.

To obtain supportive data, purified AT5G05600 was incubated with phenanthrene as a substrate, followed by an extraction of the organic phase, and then the samples were subjected to HPLC analysis (Figure 9). The assay without the enzyme served as a control (Figure 9A). When 6 µg of the enzyme were used in the reaction, the phenanthrene peak on the HPLC plot was significantly reduced in height (Figure 9B). This demonstrated that the recombinant protein enzymatically degrades this PAH *in vitro* and supports the hypothesis that phenanthrene is biodegraded by AT5G05600 *in vivo*.

3.7 AT5G05600 is a putative *Arabidopsis thaliana* flavonol synthase

An alignment of the translated ORF of *At5g05600* to flavonol synthase from both *Citrus unshui* and a previously characterized flavonol synthase *At5g08640* from *Arabidopsis thaliana* indicates

a 38% identity at the amino acid level [62] (Figure 10). Amino acid sequence analysis of AT5G05600 shows the presence of two highly conserved domains among 2-oxoglutarate- and Fe(II)-dependent dioxygenases [63]. The N-terminal domain contains Gly 93 and His 100 which are highly conserved amino acids in 2-oxoglutarate dependent dioxygenases [64]. The C-terminal domain contains His 244, Asp 246 and His 301, which are inferred residues for binding to ferrous iron (II), while Arg 311 and Ser 313 are predicted residues for binding to 2-oxoglutarate. The C-terminal domain also contains Pro 230 and Gly 285, highly conserved amino acids in 2-oxoglutarate dependent dioxygenases [63]. Thus, bioinformatics analysis shows that *At5g05600* contains coding sequences highly conserved among 2-oxoglutarate- and Fe(II)-dependent dioxygenase genes.

4. Discussion

In this report, we hypothesized that plant DOXs are part of the enzymatic system involved in chemical detoxification, in a similar manner to some bacterial DOXs. Herein we focused on *Arabidopsis thaliana* AT5G05600 and its role in the degradation of phenanthrene once internalized by the plant. Microarray analysis of 21 day-old plants continuously grown on 0.25 mM phenanthrene showed an up-regulation of *At5g05600* expression by 5.02-fold [34]. After the exposure of the reporter line GT_5_96064 to phenanthrene, GUS expression driven by the *At5g05600* promoter was localized to the cotyledons and true leaves, where vascular tissue expression was specifically noticeable (Figure 2). Given that *At5g05600* expression is elevated in response to phenanthrene exposure, we propose that the AT5G05600 protein plays an important role in phenanthrene detoxification and/or dealing with PAH-induced cellular stress. Related, global analysis of *Arabidopsis* grown in media containing 0.5 mM of the

explosive hexahydro-1,3, 5-trinitro-1,3,5-triazine (RDX) for 9 days showed that *At5g05600* expression increased by 2.80-fold [65]. Rao et al. [65] suggested a role for oxidoreductases during the first phase of explosive detoxification. Moreover, microarray analysis of salt stress responses, fungal exposure and wounding showed that *At5g05600* is up-regulated [66]. Taken together, the above results suggest possible multiple roles of AT5G05600 in metabolism during various environmentally stressful conditions.

The phenotypic stress response to PAH exposure by *Arabidopsis* includes poor root and shoot growth and altered development [32, 33, 39, 67]. Our results show that the *At5g05600* loss-of-function mutant lines exhibited a less sensitive phenotype under phenanthrene treatment (Figure 3). Conversely, *At5g05600* over-expressing plant lines were highly sensitive to phenanthrene treatments, exhibiting reduced root length (Figure 4) and foliage area (Table 1). Therefore, these genetic and physiological data suggest a role for AT5G05600 in the biotransformation of phenanthrene *in vivo*, possibly in the initial catabolism of PAHs. Toxin transformation reactions involve reduction or oxidation of toxic compounds, sometimes adding hydroxyl or carboxyl groups [46]. The products of those reactions vary in complexity and in some cases produce more toxic and harmful intermediates than the parent compound [68-70]. Our *in planta* results suggest that *At5g05600*-dependent transformation reactions convert phenanthrene into toxic metabolites, possibly saturating the PAH detoxification system such as when AT5G05600 is over-produced. Subsequently, the high levels of harmful metabolites in combination with increased ROS levels result in general growth inhibition.

In addition to transformation, toxins may be conjugated to glucose, malonyl, or glutathione by transferases, transforming them into more water-soluble compounds [30, 69, 71-75] that are then

translocated into the vacuole or cell wall compartments [69, 76]. Interestingly in *Arabidopsis*, the expression of 12 glutathione transferases increase under phenanthrene exposure, including *At4g02520*, *At2g02930*, *At1g02920*, and *At1g02930* [34, 77]. In a search for transporters, *AtNAP2* (*POP1*), a NAP-type ABC transporter which encodes a P-loop containing nucleoside triphosphate hydrolase, was up-regulated during PAH stress [34]. **Consequently, it is likely that after their transformation harmful breakdown products are sequestered in the vacuole or cell wall interfering less with cellular metabolism [48].**

To better understand the role of AT5G05600 in the detoxification of phenanthrene, *in vitro* biochemical assays were performed using the purified recombinant protein and phenanthrene as a substrate. **The data presented here suggest activity of AT5G05600 towards phenanthrene (Figure 8, 9).** The changes observed in the fluorescent emission spectrum and in relative abundance levels of phenanthrene after the incubating it with purified AT5G05600 suggest chemical structure modifications by AT5G05600. While it may not be the endogenous role for this enzyme, these results strongly indicate that AT5G05600 is using phenanthrene as a substrate and chemically transforming it. Consequently, we propose that after entering the plants, phenanthrene is oxidized initially via a hydroxylation reaction. Nevertheless, future experiments to isolate and identify possible metabolites of the reaction are needed to obtain a better understanding of the detoxification steps.

Oxidative stress is a cellular response that has been associated with abiotic and biotic stresses. In our study, the over-expressing lines exposed to phenanthrene showed more oxidative damage than wild type plants, as evident by higher **H₂O₂** levels indicated by DAB staining (Figure 6). Conversely, the *At5g05600* loss-of-function mutant lines exposed to

phenanthrene showed a substantially lower accumulation of H_2O_2 than control plants, suggesting less oxidative damage (Figure 5). ROS-induced oxidative stress adversely affects plant growth and yield, impacting photosynthetic capacity for example [78-85]. On the other hand, it is unclear which mechanisms are responsible for H_2O_2 production in PAH-induced stress. Diverse enzymes, including NADPH-dependent oxidases, amine oxidases and pH-dependent cell wall peroxidases have been implicated in ROS production [42, 86, 87]. Alternatively, it is also possible that H_2O_2 could be produced by AT5G05600 itself during the oxidation of the toxin. Nevertheless, future research is needed to investigate the sources of ROS under phenanthrene exposure.

Owens et al. [62] identified a family of six flavonol synthase (FLS) genes in the *Arabidopsis* genome. One of these, *At5g08640* (*ATFLS1*) was shown with enzymatic activity catalyzing the formation of flavonols from dihydroflavonols. Amino acid sequence analysis of AT5G05600 indicates a 38% of identity to *ATFLS1* and flavonol synthase from *Citrus unshui* (Figure 10), including key regulatory elements found in the dioxygenase superfamily [63, 64]. According to Prescott and John [47] 2-oxoacid dependent dioxygenases with more than 32% amino acid sequence identity generally perform similar functions. Thus, the bioinformatics analysis presented here suggests that AT5G05600 is a putative flavonol synthase in *Arabidopsis thaliana*. Interestingly, four of the six DOXs up-regulated under phenanthrene treatment are proposed to function in the flavonoid biosynthesis pathways [34]. In similar *Arabidopsis* experiments under naphthalene exposure, flavonoid biosynthesis genes were also up-regulated [67]. Therefore, flavonoid production may be key to dealing with PAH-induced stress considering that flavonoids are known secondary metabolites with antioxidant activity [88-92].

Still, it remains unclear if AT5G05600 is a flavonol synthase. Further studies to analyze levels and composition of flavonoids in loss-of-function and gain-of-function mutants could resolve this point. Additionally, knowing the flavonoid levels may help explain the phenotypes related to ROS production in these genetic backgrounds. It is possible that AT5G05600 is produced in response to oxidative stress and that phenanthrene catabolism is a by-product of that induction because AT5G05600 shows substrate promiscuity.

Plant xenobiotic detoxification is a multi-enzyme multi-step process. The discovery of novel genes involved in the uptake, transport, transformation, conjugation and storage of PAHs will enable phytoremediation development [13]. In addition, novel genetic engineering approaches should be explored in the design of transgenic plants capable of removing and/or biodegrade PAHs. For instance, our studies revealed that the *At5g05600* loss-of-function mutant lines are more tolerant to PAHs than control plants, which could mean uptake without degradation. Because the loss-of-function DOX plants are less affected by PAHs, such as producing less H₂O₂ (Figure 5), plants lacking this enzyme could potentially be used to hyperaccumulate and remove PAHs as a bioremediation strategy, although phenanthrene concentrations in tissues need to be further characterized. Alternatively, the concomitant over-expression of enzymatic and/or non-enzymatic pathways for ROS scavenging [93-95] in the DOX over-expressing plants should dampen the oxidative stress experienced by plants due to PAH exposure. Therefore, a combinatorial approach to deal the oxidative stress, as well as PAH degradation, is recommended.

In summary, we provide evidence to show that AT5G05600, a putative flavonol synthase, is part of the phenanthrene-induced stress response and probably involved in the first detoxification phase for PAHs in *Arabidopsis*. Data from both *in vivo* and *in vitro* experiments show that this enzyme has an activity towards phenanthrene, changing its structure. We propose that other PAHs follow a route of detoxification, similar to that of phenanthrene. These findings indicate possible strategies involving multi-transgene technologies that target catabolic pathways able to degrade recalcitrant PAHs while addressing the oxidative stress associated with the detoxification.

Acknowledgements

We thank Dr. Katherine Gibson and Dr. Steve Ackerman for the original review of this manuscript. This work was supported by the University of Massachusetts Boston and a Joint Interagency Program on Phytoremediation Research grant from the National Science Foundation (IBN-0343856) to AC-C.

References

- 1) R. G. Harvey, Polycyclic aromatic hydrocarbons: chemistry and carcinogenicity, first ed., Cambridge University Press, Cambridge, UK, 1991.
- 2) D. Phillips, Fifty years of benzo[a]pyrene, *Nature* 303 (1983) 468-472.
- 3) W.M. Baird, L.A. Hooven, B. Mahadevan, Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action, *Environ. Mol. Mutagen.* 45 (2005) 106-114.
- 4) A.R. Johnsen, L.Y. Wick, H. Harms, Principles of microbial PAH-degradation in soil, *Environ. Pollut.* 133 (2005) 71-84.
- 5) S.C. Edwards, W. Jedrychowski, M. Butscher, D. Camann, A. Kieltyka, E. Mroz, E. Flak, Z. Li, S. Wang, F.P. Perera, Prenatal exposure to polycyclic aromatic hydrocarbons (PAHs) negatively affects children's neurodevelopment at 5 years of age in an inner-city prospective cohort in Krakow (Poland), *Environ. Health Perspect.* 118 (2010) 1326-1331.
- 6) D.L. Diggs, A.C. Huderson, K.L. Harris, J.N. Myers, L.D. Banks, P.V. Rekhadevi, M.S. Niaz, A. Ramesh, Polycyclic aromatic hydrocarbons and digestive tract cancers: a perspective, *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.* 9 (2011) 324-357.
- 7) Office of the Federal Register. Appendix A –126 Priority Pollutants. Federal Register, 1982, 47: 52309.8.
- 8) A. El Amrani, A.S. Dumas, L. Wick, E. Yergeau, R. Berthomé, "Omics" insights into PAH degradation toward improved green remediation biotechnologies, *Environ. Sci. Technol.* 49 (2015) 11281-11291.
- 9) P. C. Abhilash, S. Jamil, N. Signh, Transgenic plants for enhanced biodegradation and phytoremediation for organic xenobiotics, *Biotech. Adv.* 27 (2009) 474-488.
- 10) M. Megharaj, B. Ramakrishnan, B., Ramakrishnan, K. Venkateswarlu, N. Sethunathan, R. Naidu, Bioremediation approaches for organic pollutants: a critical perspective, *Environ. Int.* 37 (2011) 11-41.
- 11) E. Pilon-Smits, Phytoremediation, *Annu. Rev. Plant Biol.* 56 (2005) 15-39.
- 12) S.L. Doty, Enhancing phytoremediation through the use of transgenics and endophytes, *New Phytol.* 179 (2008) 318-333.
- 13) K. Gerhardt, X.D. Huang, B.R. Glick, B.M. Greenberg, Phytoremediation and rhizoremediation of organic soil contaminants: potential and challenges, *Plant Sci.* 176 (2009) 20-30.
- 14) H. Ali, E. Khan, M.A. Sajad, Phytoremediation of heavy metals-concepts and applications, *Chemosphere* 91 (2013) 869-881.
- 15) H. Sandermann, Plant metabolism of xenobiotics, *TIBS* 17 (1992) 82-84.

- 16) A.C. Dietz, J.L. Schnoor, Advances in phytoremediation, *Environ. Health Perspect.* 109 (2001) 163-168.
- 17) Q. Chaudhry, M. Blom-Zandstra, S. Gupta, E.J. Joner, Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment, *Environ. Sci. Pollut. R.* 12 (2005) 34-48.
- 18) T. Mithaishvili, R. Scalla, D. Ugrekhelidze, B. Tsereteli, T. Sadunishvili, G. Kvesitadze, Degradation of aromatic compounds in plants grown under aseptic conditions, *Z. Naturforsch. C* 60 (2005) 97-102.
- 19) W. Wenzel, Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils, *Plant Soil* 321 (2009) 385-408.
- 20) X.Z. Yu, S.C. Wu, F.Y. Wu, M.H. Wong, Enhanced dissipation of PAHs from soil using mycorrhizal ryegrass and PAH-degrading bacteria, *J. Hazard. Mater.* 186 (2011) 1206–1217.
- 21) V. D’Orazio, A. Ghanem, N. Senesi, Phytoremediation of pyrene contaminated soils by different plant species, *Clean Soil Air Water* 41 (2013) 377-382.
- 22) J. Chen, Q. X. Xu, Y. Su, Z.Q. Shi, F. Han, Phytoremediation of organic polluted soil, *J. Bioremed. Biodeg.* 4 (2013) e132. doi:10.4172/2155-6199.1000e132.
- 23) P. Harvey, B. Campanella, P. Castro, H. Harms, E. Lichtfouse, A. Schäffner, S. Smrcek, D. Werck-Reichhart, Phytoremediation of polyaromatic hydrocarbons, anilines and phenols, *Environ. Sci. Pollut. Res. Int.* 9 (2002) 29–47.
- 24) O.V. Singh, R.K. Jain, Phytoremediation of toxic aromatic pollutants from soil, *Appl. Microbiol. Biotechnol.* 63 (2003) 128-135.
- 25) S. Kathi, B. Khan, Phytoremediation approaches to PAH contaminated soil, *Indian J. Sci. Technol.* 4 (2011) 56-63.
- 26) T. Ishikawa, The ATP-dependent glutathione S-conjugate export pump, *TIBS* 17 (1992) 463–469.
- 27) Y.-P. Lu, Z.-S. Li, P.A. Rea, AtMRP1 of *Arabidopsis thaliana* encodes a glutathione S-conjugate pump: Isolation and functional definition of a plant ATP-binding cassette transporter gene, *Proc. Natl. Acad. Sci. USA* 94 (1997) 8243–8348.
- 28) T. Ishikawa, Z.S. Li, Y.P. Lu, P.A. Rea, The GS-X pump in plant, yeast, and animal cells: structure, function, and gene expression, *Biosci. Rep.* 17 (1997) 189–207.
- 29) A. Frelet-Barrand, H.U. Kolukisaoglu, S. Plaza, M. Ruffer, L. Azevedo, S. Hörtensteiner, K. Marinova, B. Weder, B. Schulz, M. Klein, Comparative mutant analysis of Arabidopsis ABCC-Type ABC transporters: AtMRP2 contributes to detoxification, vacuolar organic anion transport and chlorophyll degradation, *Plant Cell Physiol.* 49 (2008) 557-569.
- 30) S. Pang, L. Duan, Z. Liu, X. Song, X. Li, C. Wang, Co-Induction of a glutathione-S-transferase, a glutathione transporter and an ABC transporter in maize by xenobiotics, *PLoS ONE* 7 (2012) e40712. doi:10.1371/journal.pone.0040712

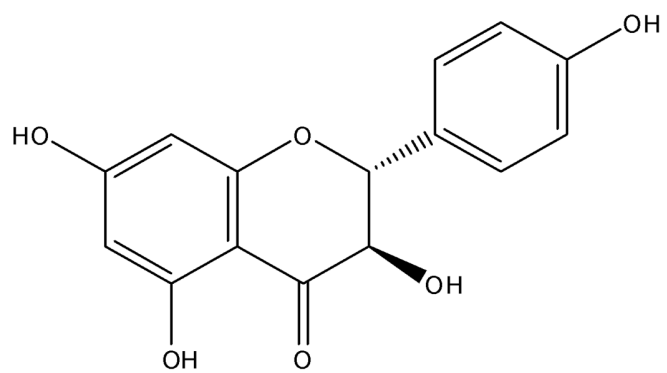
- 31) E. Remy, P. Duque, Beyond cellular detoxification: A plethora of physiological roles for MDR transporter homologs in plants, *Front. Physiol.* 5 (2014) 201.
doi:10.3389/fphys.2014.00201
- 32) M. Alkio, T. Tabuchi, X. Wang, A. Colón-Carmona, Stress responses to polycyclic aromatic hydrocarbons in *Arabidopsis* include growth inhibition and hypersensitive response-like symptoms, *J. Exp. Bot.* 56 (2005) 2983-2994.
- 33) H. Liu, D. Weisman, Y. Ye, B. Cui, Y. Huang, A. Colón-Carmona, Z. Wang, An oxidative stress response to polycyclic aromatic hydrocarbon exposure is rapid and complex in *Arabidopsis thaliana*, *Plant Sci.* 176 (2009) 375-382.
- 34) D. Weisman, M. Alkio, A. Colon-Carmona, Transcriptional responses to polycyclic aromatic hydrocarbon-induced stress in *Arabidopsis thaliana* reveal the involvement of hormone and defense signaling pathways, *BMC Plant Biol.* 10 (2010) 59.
doi:10.1186/1471-2229-10-59.
- 35) A. Jajoo, N.R. Rao Mekala, R.S. Tomar, M. Grieco, M. Tikkanen, E.M. Aro , Inhibitory effects of polycyclic aromatic hydrocarbons (PAHs) on photosynthetic performance are not related to their aromaticity, *J. Photochem. Photobiol. B137* (2014) 151–155.
doi:10.1016/j.jphotobiol.2014.03.011
- 36) C. A. Marwood, K.T. Bestari, R.W. Gensemer, K.R. Solomon, B.M. Greenberg, Creosote toxicity to photosynthesis and plant growth in aquatic microcosms, *Environ. Toxicol. Chem.* 22 (2003) 1075-1085.
- 37) V. Pakova, K. Hilscherova, M. Feldmannova, L. Blaha, Toxic effects and oxidative stress in higher plants exposed to polycyclic aromatic hydrocarbons and their N-heterocyclic derivatives, *Environ. Toxicol. Chem.* 25 (2006) 3238-3245.
- 38) D.J Burritt, The polycyclic aromatic hydrocarbon phenanthrene causes oxidative stress and alters polyamine metabolism in the aquatic liverwort *Riccia fluitans L.*, *Plant Cell Environ.* 31(2008) 1416-1431.
- 39) H. Liu, Y.B. Ye, B. Cui, L.M. Zheng, Y.H. Huang, Z.H. Wang, Responses of *Arabidopsis thaliana* to oxidative stress induced by polycyclic aromatic hydrocarbon fluoranthene, *Ying Yong Sheng Tai Xue Bao* 19 (2008) 413-418.
- 40) G.J. Ahammed, S.P. Choudhary, S. Chen, X. Xia, K. Shi, Y. Zhou, J. Yu, Role of brassinosteroids in alleviation of phenanthrene–cadmium co-contamination induced photosynthetic inhibition and oxidative stress in tomato, *J. Exp. Bot.* 64 (2012) 199-213.
- 41) G.J. Ahammed, C.J. Gao, J.O. Ogwen, Y.H. Zhou, X.J. Xia, W.H. Mao, K. Shi, J.Q. Yu, Brassinosteroids induce plant tolerance against phenanthrene by enhancing degradation and detoxification in *Solanum lycopersicum L.*, *Ecotoxicol. Environ. Safety* 80 (2012) 28-36.
- 42) S. Choudhury, P. Panda, L. Sahoo, S.K. Panda, Reactive oxygen species signaling in plants under abiotic stress, *Plant Signal. Behav.* 8 (2013) e23681.
doi:10.4161/psb.23681.

- 43) H. Wei, S. Song, H. Tian, T. Liu, Effects of phenanthrene on seed germination and some physiological activities of wheat seedling, *C.R. Biol.* 337 (2014) 95-100.
- 44) R. Kanaly, S. Harayama, Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria, *J. Bacteriol.* 182 (2000) 2059–2067.
- 45) R. Parales, K. Lee, S. Resnick, H. Jiang, D. Lessner, D. Gibson, Substrate specificity of naphthalene dioxygenase: effect of specific amino acids at the active site of the enzyme, *J. Bacteriol.* 182 (2000) 1641-1649.
- 46) T. Macek, M. Mackova, J. Kas, Exploitation of plants for the removal of organics in environmental remediation, *Biotech. Adv.* 18 (2000) 23-34.
- 47) A.G. Prescott, P. John, Dioxygenases: molecular structure and role in plant metabolism, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47 (1996) 245–271.
- 48) R.B. Meagher, Phytoremediation of toxic elemental and organic pollutants, *Curr. Opin. Plant Biol.* 3 (2000) 153–162.
- 49) K.W. Earley, J.R. Haag, O. Pontes, K. Oppen, T. Juehne, K. Song, C.S. Pikaard, Gateway-compatible vectors for plant functional genomics and proteomics, *Plant J.* 45 (2006) 616–629.
- 50) R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, Gus fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants, *EMBO J.* 6 (1987) 3901-3907.
- 51) H. Thordal-Christensen, Z. Zhang, Y. Wei, D. Collinge, Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction, *Plant J.* 11 (1997) 1187–1194.
- 52) L. Britsch, H. Grisebach, Purification and characterization of (2S)-flavanose 3-hydroxylase from *Petunia hybrida*, *Eur. J. Biochem.* 156 (1986) 569-577.
- 53) N. Wu, S. Zhang, H. Huang, P. Christie, Enhanced dissipation of phenanthrene in spiked soil by arbuscular mycorrhizal alfalfa combined with a non-ionic surfactant amendment, *Sci. Total Environ.* 394 (2008) 230-236.
- 54) X. Zhang, R. Henriques, S.S. Lin, Q.W. Niu, N.H. Chua, *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method, *Nat. Protoc.* 1 (2006) 641-646.
- 55) A. Marchler-Bauer, C. Zheng, F. Chitsaz, M.K. Derbyshire, L.Y. Geer, R.C. Geer, N.R. Gonzales, M. Gwadz, D.I. Hurwitz, C.J. Lanczycki, F. Lu, S. Lu, G.H. Marchler, J.S. Song, N. Thanki, R.A. Yamashita, D. Zhang, S.H. Bryant, CDD: conserved domains and protein three-dimensional structure, *Nucleic Acids Res.* 41 (2013) D348-352.
- 56) S. Kertbundit, H. De Greve, F. Deboeck, M. Van Montagu, J.P. Hernalsteens, *In-vivo* random β -glucuronidase gene fusions in *Arabidopsis thaliana*, *Proc. Natl. Acad. Sci. USA* 88 (1991) 5212–5216.
- 57) J.T. Odell, F. Nagy, N.H. Chua, Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter, *Nature* 313 (1985) 810-812.
- 58) P.N. Benfey, N.H. Chua, The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants, *Science* 250 (1990) 959-966.

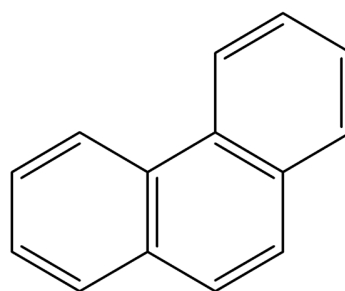
- 59) R. Okparanma, A. Mouazen, Determination of total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) in soils: a review of spectroscopic and non-spectroscopic techniques, *Appl. Spectrosc. Rev.* 48 (2013) 458-486.
doi: 10.1080/05704928.2012.736048
- 60) J. Aldstadt, R. St. Germain, T. Grundl, R. Schweitzer, An in situ laser-induced fluorescence system for polycyclic aromatic hydrocarbon-contaminated sediments, United States Environmental Agency, Great Lakes National Program Office, Chicago, Illinois, 2002.
- 61) R. Dabestani, I. Ivanov, A compilation of physical, spectroscopic and photophysical properties of polycyclic aromatic hydrocarbons, *Photochem. Photobiol.* 70 (1999) 10-24.
- 62) D.K. Owens, A.B. Alerding, K.C. Crosby, A.B. Bandara, J.H. Westwood, B.S. Winkel, Functional analysis of a predicted flavonol synthase gene family in *Arabidopsis*, *Plant Physiol.* 147 (2008) 1046-1061.
- 63) G.Z. Lin, Y.J. Lian, J.H. Ryu, M.K. Sung, J.S. Park, H.J. Park, B.K. Park, J.S. Shin, M.S. Lee, C.I. Cheon, Expression and purification of His-tagged flavonol synthase of *Camellia sinensis* from *Escherichia coli*, *Protein Express. Purif.* 55 (2007) 287-292.
- 64) S.C. Farrow, P.J. Facchini, Functional diversity of 2-oxoglutarate/Fe(II)-dependent dioxygenases in plant metabolism, *Front. Plant Sci.* 5 (2014) 524. doi: 10.3389/fpls.2014.00524
- 65) M.R. Rao, M.D. Halfhill, L.G. Abercrombie, P. Ranjan, J.M. Abercrombie, J.S. Gouffon, A.M. Saxton, C.N. Steward, Phytoremediation and phytosensing of chemical contaminants, RDX and TNT: identification of the required target genes, *Funct. Integr. Genomics* 9 (2009) 537-547.
- 66) J. Kilian, D. Whitehead, J. Horak, D. Wanke, S. Weinl, O. Batistic, C. D'Angelo, E. Bornberg-Bauer, J. Kudla, K. Harter, The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses, *Plant J.* 50 (2007) 347-363.
- 67) R.H. Peng, R.R. Xu, X.Y. Fu, A.S. Xiong, W. Zhao, Y.S. Tian, B. Zhu, X.F. Jin, C. Chen, H.J. Han, Q.H. Yao, Microarray analysis of the phytoremediation and phytosensing of occupational toxicant naphthalene, *J. Hazard. Mater.* 189 (2011) 19-26.
- 68) K.L. Shuttleworth, C.E. Cerniglia, Environmental aspects of PAH biodegradation, *Appl. Biochem. Biotechnol.* 54 (1995) 292-302.
- 69) J. Coleman, M. Blake-Kalff, E. Davies, Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation, *Trends Plant Sci.* 2 (1997) 144-151.
- 70) T.S. Babu, J.B. Marder, S. Tripuranthakam, D.G. Dixon, B. M. Greenberg, Synergistic effects of a photooxidized polycyclic aromatic hydrocarbon and copper on photosynthesis and plant growth: evidence that in vivo formation of reactive oxygen species is a mechanism of copper toxicity, *Environ. Toxicol. Chem.* 20 (2001) 1351-1358.
- 71) E.J. Belford, U. Dorfler, A. Stampfl, P. Schroder, Microsomal detoxification enzymes in yam bean (*Pachyrhizus erosus* (L.) urban), *Z Naturforsch C.* 59 (2004) 693-700.

- 72) F. Gandia-Herrero, A. Lorenz, T. Larson, I.A. Graham, D.J. Bowles, E.L. Rylott, N.C. Bruce, Detoxification of the explosive 2,4,6-trinitrotoluene in *Arabidopsis*: discovery of bifunctional O- and C-glucosyltransferases, *Plant J.* 56 (2008) 963–974.
- 73) S. Mohsenzadeh, M. Esmaili, F. Moosavi, M. Shahrtash, B. Saffari, H. Mohabatkar, Plant glutathione S-transferase classification, structure and evolution, *Afr. J. Biotechnol.* 10 (2011) 8160-8165.
- 74) M. Shahrtash, Plant glutathione S-transferases function during environmental stresses: a review article, *Rom. J. Biol. – Plant Biol.* 58 (2012) 19-25.
- 75) A. San Miguel, P. Schröder, R. Harpaintner, T. Gaude, P. Ravanel, M. Raveton, Response of phase II detoxification enzymes in *Phragmites australis* plants exposed to organochlorines, *Environ. Sci. Pollut. Res. Int.* 20 (2013) 3464-3471.
- 76) K.A. Marrs, M.R. Alfenito, A.M. Lloyd, V. Walbot, A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene Bronze-2, *Nature* 375 (1995) 397–400.
- 77) H. Liu, D. Weisman, L. Tang, L. Tan, W.K. Zhang, Z.H. Wang, Y.H. Huang, W.X. Lin, X.M. Liu, A. Colón-Carmona, Stress signaling in response to polycyclic aromatic hydrocarbon exposure in *Arabidopsis thaliana* involves a nucleoside diphosphate kinase, NDPK-3, *Planta* 241 (2014) 95-107. doi: 10.1007/s00425-014-2161-8
- 78) C.H. Foyer, S. Shigeoka, Understanding oxidative stress and antioxidant functions to enhance photosynthesis, *Plant Physiol.* 155 (2011) 93–100.
- 79) J.J. Slaski, D.J. Archambault, X. Li, Physiological tests to measure impacts of gaseous polycyclic aromatic hydrocarbons (PAHs) on cultivated plants, *Commun. Soil Sci. Plan.* 33 (2002) 3227-3239.
- 80) M. Kummerová, J. Krulová, S. Zezulka, J. Tríska, Evaluation of fluoranthene phytotoxicity in pea plants by Hill reaction and chlorophyll fluorescence, *Chemosphere* 65 (2006) 489-496.
- 81) M. Kummerova, L. Vanova, H. Fiserova, M. Klems, S. Zezulka, J. Krulova, Understanding the effect of organic pollutant fluoranthene on pea in vitro using cytokinins, ethylene, ethane and carbon dioxide as indicators, *Plant Growth Regul.* 61 (2010) 161-174.
- 82) R. Singh-Tomar, A. Jajoo, Alteration in PS II heterogeneity under the influence of polycyclic aromatic hydrocarbon (fluoranthene) in wheat leaves (*Triticum aestivum*), *Plant Sci.* 209 (2013) 58-63.
- 83) A. Przybysz, R. Popek, H. Gawrońska, K. Grab, K. Łoskot, M. Wrochna, S. Gawroński, Efficiency of photosynthetic apparatus of plants grown in sites differing in level of particulate matter, *Acta Sci. Pol.* 13 (2014) 17-30.
- 84) S.A. Hasan, S. Hayat, A. Ahmad, Brassinosteroids protect photosynthetic machinery against the cadmium induced oxidative stress in two tomato cultivars, *Chemosphere* 84 (2011) 1446–1451.

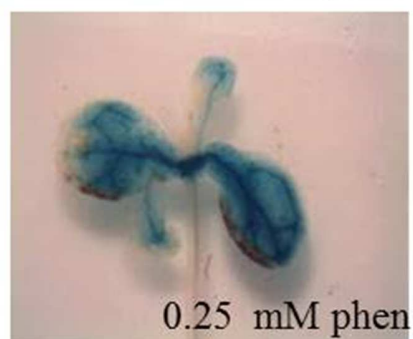
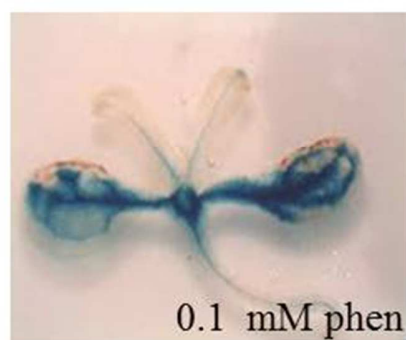
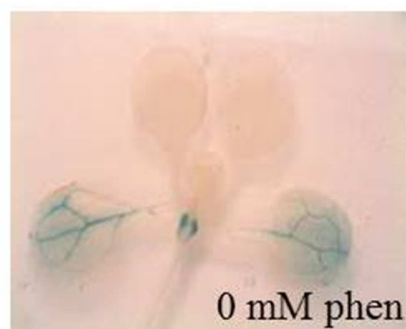
- 85) G.J. Ahammed, H.L. Yuan, J.O. Ogwen, Y.H. Zhou, X.J. Xia, W.H. Mao, K. Shi, J.Q. Yu, Brassinosteroid alleviates phenanthrene and pyrene phytotoxicity by increasing detoxification activity and photosynthesis in tomato, *Chemosphere* 86 (2012) 546–555.
- 86) H. Wohlgemuth, K. Mittelstrass, S. Kschieschan, J. Bender, H.J. Weigel, K. Overmyer, J. Kangasjarvi, H. Sandermann, C. Langebartels, Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone, *Plant Cell Environ.* 25 (2002) 717-726.
- 87) R. Mittler, S. Vanderauwera, N. Suzuki, G. Miller, V.B. Tognetti, K. Vandepoele, M. Gollery, V. Shulaev, F.V. Breusegem, ROS signaling: the new wave?, *Trends Plant Sci.* 16 (2011) 300-309.
- 88) J.A. Vinson, Y.A. Dabbagh, M. Serry, J. Jinhee, Plant flavonoids, especially tea flavonols, are powerful antioxidants using an *in vitro* oxidation model for heart disease, *J. Agricult. Food Chem.* 43 (1995) 2800–2802.
- 89) C. Rice-Evans, Flavonoid antioxidants, *Curr. Med. Chem.* 8 (2001) 797–807.
- 90) S.J.S. Flora, Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure, *Oxid. Med. Cell. Longev.* 2 (2009) 191-206.
- 91) C. Buer, N. Imin, M. Djordjevic, Flavonoids: new roles for old molecules, *J. Integr. Plant Biol.* 52 (2010) 98-111.
- 92) G. Agati, E. Azzarello, S. Pollastri, M. Tattini, Flavonoids as antioxidants in plants: Location and functional significance, *Plant Sci.* 196 (2012) 67–76.
- 93) D.M. Xi, W.S. Liu, G.D. Yang, C.A. Wu, C.C. Zheng, Seed-specific overexpression of antioxidant genes in *Arabidopsis* enhances oxidative stress tolerance during germination and early seedling growth. *Plant Biotechnol. J.* 8 (2010) 796-806.
- 94) C.G. Flocco, S.D. Lindblom, E. Pilon Smits, Overexpression of enzymes involved in glutathione synthesis enhances tolerance to organic pollutants in *Brassica juncea*. *Int. J. Phytoremediation* 6 (2004) 1–16.
- 95) V. Kumar, S.K. Yadav, Overexpression of CsDFR and CsANR enhanced flavonoids accumulation and antioxidant potential of roots in tobacco, *Plant Root* 7 (2013) 65-76. doi:10.3117/plantroot.7.65

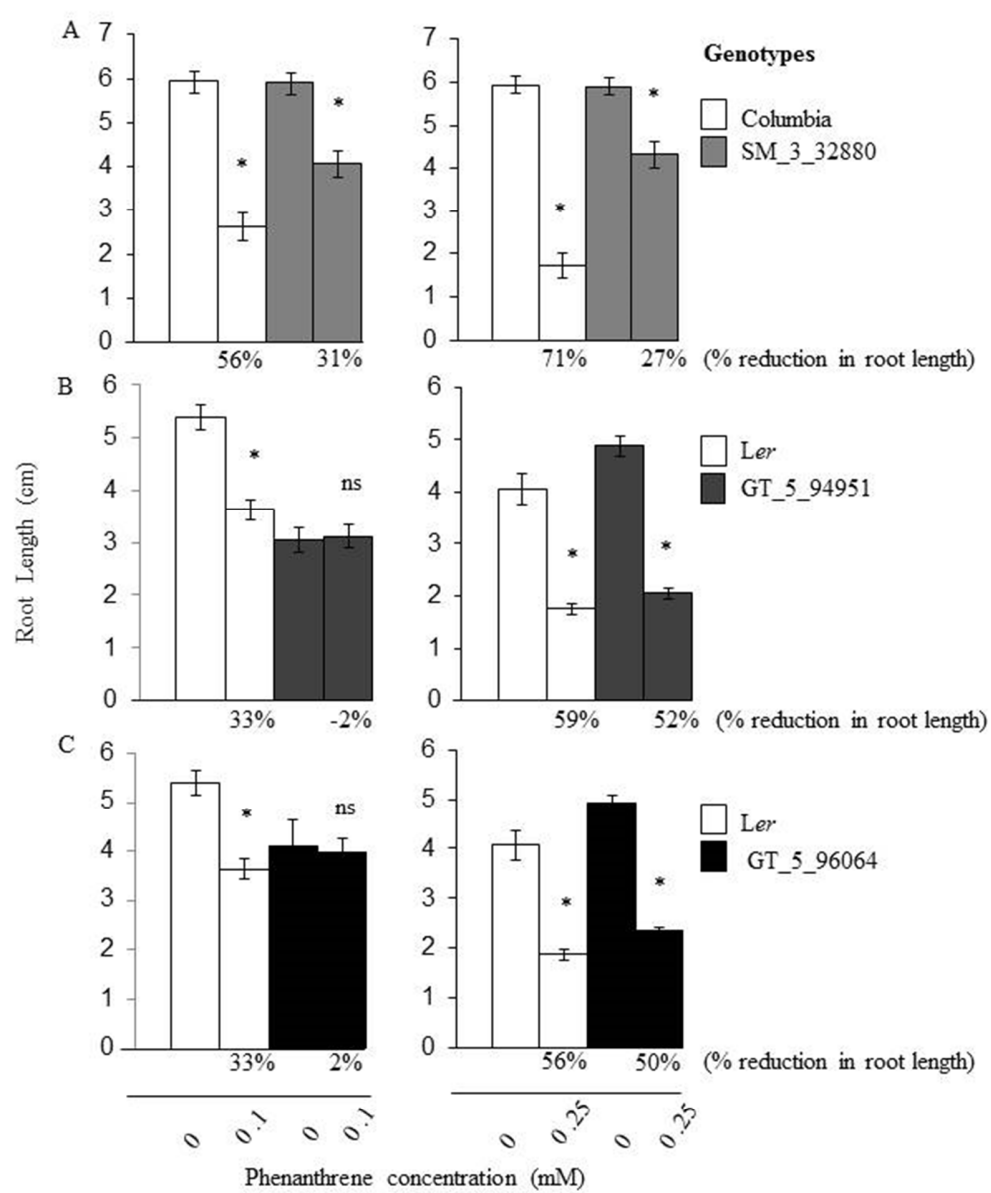


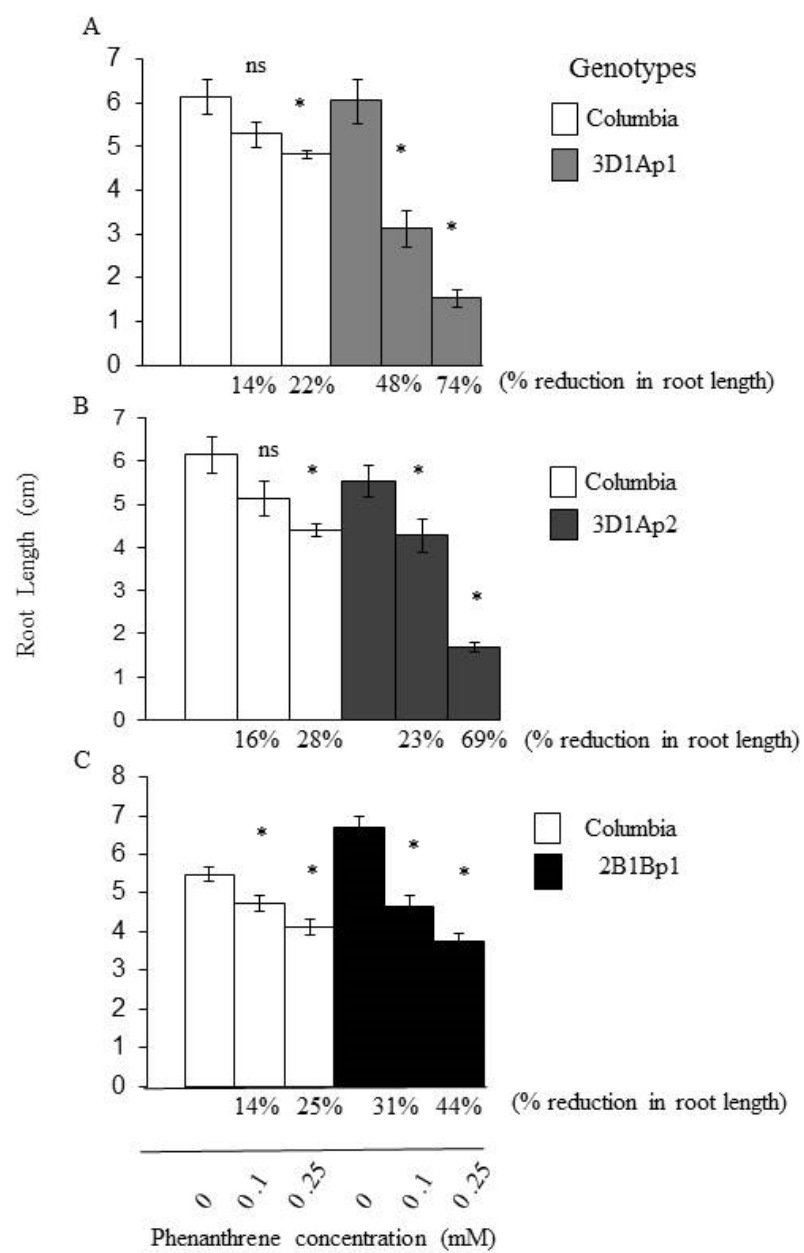
2R, 3R dihydrokaempferol

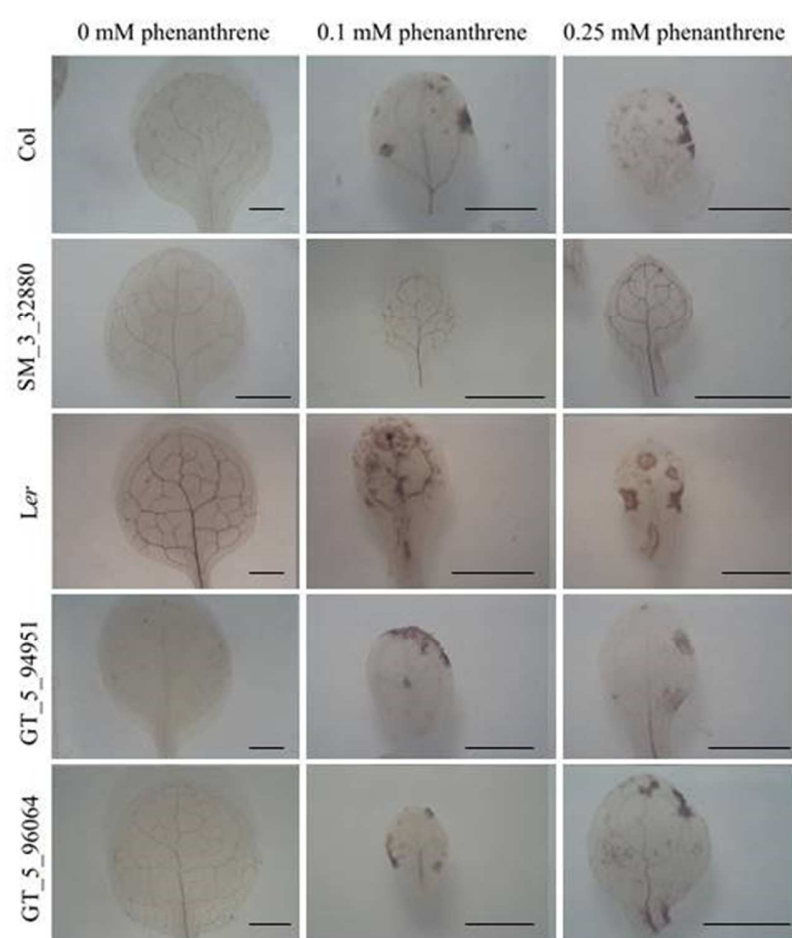


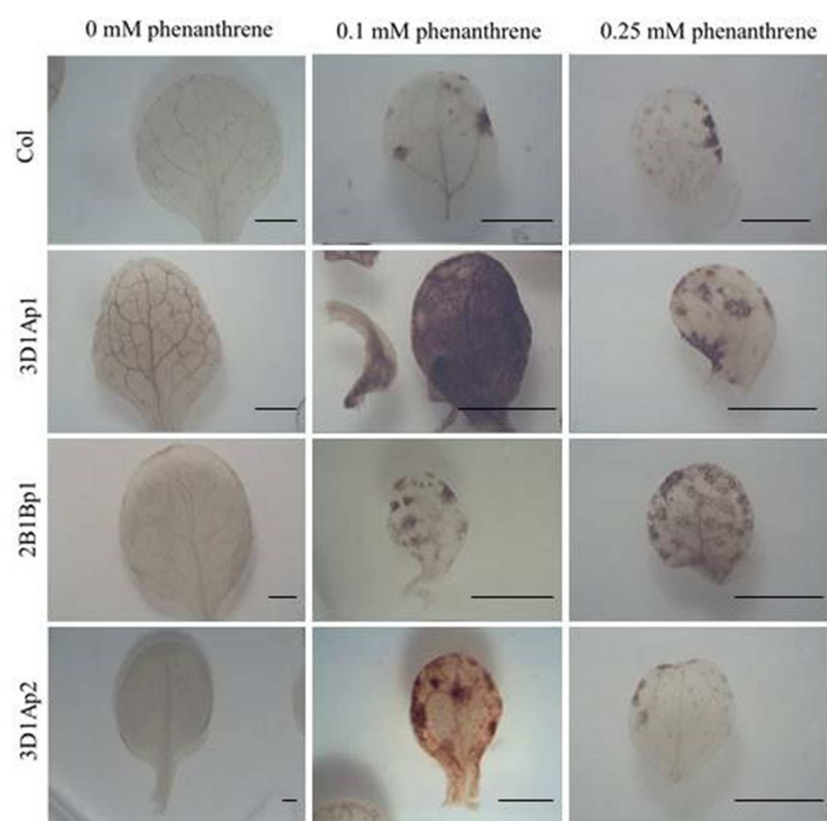
Phenanthrene

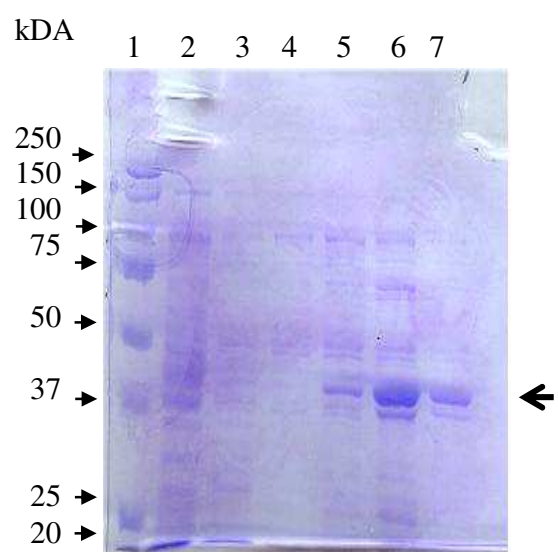


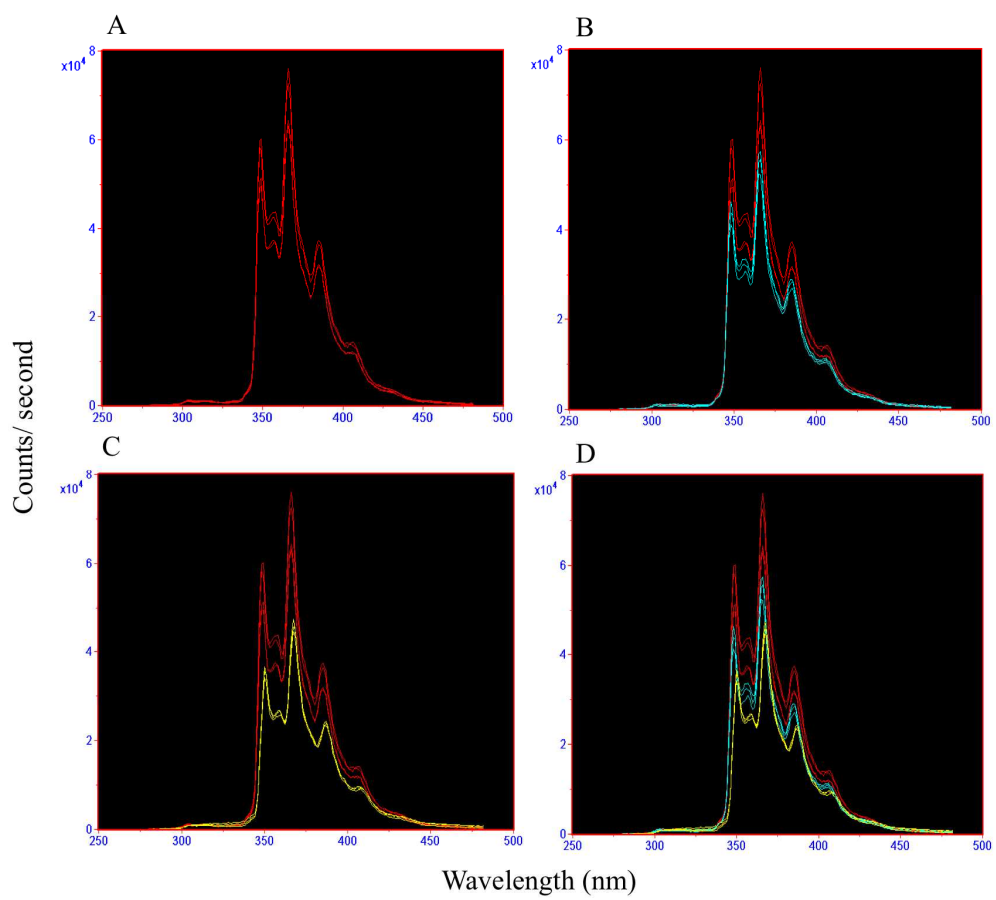


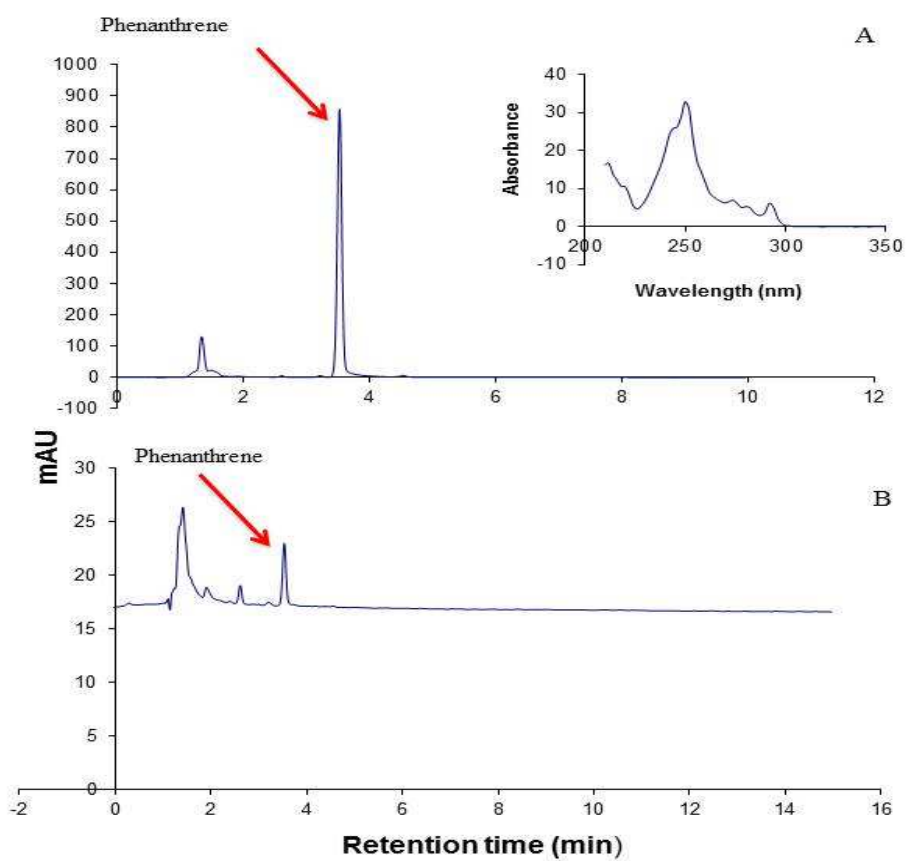












AT5G08640	-----MEVERVQDISSSLLTEAIPLEFIRSEKEQPAITTFRGP	39
Citrus	-----MEVERVQAIASLSHNGTIPAEFIRPEKEQPASTTTHGP	39
AT5G05600	MNKNKIDVKIETKKGSMDEWPEPIVRVQSLAESNLS--SLPDRYIKPASLRPTTTEDAPT	58
	*** * *	
AT5G08640	TPAIPVVDLS-----DPDEESVRRRAVVKASEEWGLFQVNVHGIPTTELIRRLQDVGRKFF	93
Citrus	APEIPTIDLD-----DPVQDRLVRSIAEASREWGIFQVTNHGIPSDLICKLQAVGKEFF	93
AT5G05600	ATNIPIIDLEGLFSEEGLSDDVIMARISEACRGWGFFQVNVHGVKPELMDAARENWREFF	118
	** ** *	** ** *
AT5G08640	ELPSSEKESVAKPEDSKDIEGYGTLKQKDPGKKAWVDHLFHRWPPSCVNYRFWPKNPP	153
Citrus	ELPQEEKEVYSRPADAKDVQGYGTLKQKEVEGKKSVDHLFHRVWPPSSINRFWPKNPP	153
AT5G05600	HMPVNAKETYSN--SPRTYEGYGSRLGVEKGASLDWSDYYFLHLLPHHLKDFNKWPSFPP	176
	* **	* * * *
AT5G08640	EYREVNEEYAVHVKKLSETLLGILSDGLGLKRDALKEGLGGEMAEYMMKINYYP	213
Citrus	SYRAVNEEYAKYMREVVDKLFYTLSLGLGVEGGVLKEAAGGDDIEYMLKINYYP	213
AT5G05600	TIREVIDEYGEELVKLSGRIMRVLSTNLGLKEDKFQEAFFGENIGACLRVNYYPKCPRPE	236
	* * **	** ** *
AT5G08640	LALGVPAHTDLSGITLLVPN-EVPGLQVFKDDHWFDAEYIPSAVIVHIGDQILRLSNGRY	272
Citrus	LALGVVAHTDLSALTVLVPN-EVPGLQVFKDDRWDIDAKYIPNALVIHIGDQIEILSNGKY	272
AT5G05600	LALGLSPHSDPGGMTILLPDDQVFLQVRKDDTWITVKPHPHAFIVNIGDQIQILSNSTY	296
	***** *	***** *
AT5G08640	KNVLHRTTVDKETRMSSWPVFLEPPREKIVGPLPELTGDDNPPKFKPFAFKDYSYRKLNK	332
Citrus	KAVLHRTTVNKDKTRMSWPVFLEPPADTVVGPLPQLVDDENPPKYKAKKFKDYSYCKLNK	332
AT5G05600	KSVELHIVIVNSDKERVSLAFFYNPKSDIPIQLQELVSTHNPPLYPPMTFDQYRLFIRTQ	356
	* * * *	* * *
AT5G08640	LPLDKSHVESHISPR	347
Citrus	LPQ-----	335
AT5G05600	GPQG-----	360
	*	

Plant lines	Genotype	Percent reduction ^a	
		0.1 mM phen	0.25 mM phen
control	Columbia (Col)	50%	63%
	Landsberg <i>erecta</i> (Ler)	82%	83%
loss-of-function lines	SM_3_32880 (Col)	60%	65%
	GT_5_94951 (Ler)	84%	86%
	GT_5_96064 (Ler)	83%	86%
over-expression lines	3D1Ap1 (Col)	75%	76%
	3D1Ap2 (Col)	69%	73%
	2B1Bp1 (Col)	64%	76%