Transcript and protein profiling analysis of OTA-induced cell death reveals the regulation of the toxicity response process in Arabidopsis thaliana

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Abstract

Ochratoxin A (OTA) is a toxic isocoumarin derivative produced by various species of mould which mainly grow on grain, coffee, and nuts. Recent studies have suggested that OTA induces cell death in plants. To investigate possible mechanisms of OTA phytotoxicity, both digital gene expression (DGE) transcriptomic and two-dimensional electrophoresis proteomic analyses were used, through which 3118 genes and 23 proteins were identified as being up- or down-regulated at least 2-fold in Arabidopsis leaf in response to OTA treatment. First, exposure of excised Arabidopsis thaliana leaves to OTA rapidly causes the hypersensitive reponse, significantly accelerates the increase of reactive oxygen species and malondialdehyde, and enhances antioxidant enzyme defence responses and xenobiotic detoxification. Secondly, OTA stimulation causes dynamic changes in transcription factors and activates the membrane transport system dramatically. Thirdly, a concomitant persistence of compromised photosynthesis and photorespiration is indicative of a metabolic shift from a highly active to a weak state. Finally, the data revealed that ethylene, salicylic acid, jasmonic acid, and mitogen-activated protein kinase signalling molecules mediate the process of toxicity caused by OTA. Profiling analyses on Arabidopsis in response to OTA will provide new insights into signalling transduction that modulates the OTA phytotoxicity mechanism, facilitate mapping of regulatory networks, and extend the ability to improve OTA tolerance in Arabidopsis.

Key words: Cell death, OTA, proteomic, toxicity response, transcriptomic.

Introduction

In plant pathology, many secondary metabolites produced by fungi are pathogenicity or virulence factors (i.e. they play a role in causing or exacerbating plant disease; Bennett, 2003). Nectrotrophic phytopathogenic fungi synthesize a wide range of phytotoxic compounds, including the sphinganine analogue mycotoxins, which are produced by at least two unrelated groups of fungi, Alternaria and Fusarium spp. AAL toxins and fumonisins (including FB1) are sphinganine analogue mycotoxins that may play a role in virulence (Stone et al., 2000; Egusa et al., 2009). These toxins inhibit ceramide synthase, resulting in the depletion of ceramides and accumulation of free sphingoid bases. Ultimately, they induce apoptotic (or apoptotic-like) cell death in susceptible tomato cells and mammalian cells (Egusa et al., 2009; Ciacci-Zanella and Jones, 1999).

Ochratoxin A (OTA) is another naturally occurring mycotoxin produced by fungi that is found in a variety of food commodities, such as cereals, green coffee, cocoa, dried fruits, and meat products, resulting in continuous exposure of the human population to OTA (Zhang et al., 2000).
OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic, and immunotoxic to several species of animals and is known to cause kidney and liver tumours in mice and rats (Ringot et al., 2006). Several major mechanisms have been shown to be involved in the toxicity of OTA: inhibition of protein synthesis, interference with metabolic systems involving phenylalanine, promotion of membrane lipid peroxidation, disruption of calcium homeostasis, inhibition of mitochondrial respiration, and DNA damage (Ringot et al., 2006). Recent research has focused on the ability of OTA to disturb cellular signalling and regulation, as well as to modulate physiological signals, known to influence cell viability and proliferation. Recent studies have specifically focused on (i) metabolism-mediated toxicity via oxidative stress; (ii) intracellular OTA accumulation as a function of organic anion transporters; and (iii) inter- and intracellular signal transduction at nanomolar concentrations (Boesch-Saadatmandi et al., 2008; Zhang et al., 2009; Malekinejad et al., 2011).

Studies have shown that OTA is produced by phytopathogenic Aspergillus ochraceus and Aspergillus carbonarius strains, suggesting that this toxin may play a role in the aetiology of plant diseases (Xu et al., 2009). The plant response to attempted infection by microbial pathogens is often accompanied by rapid cell death in and around the initial infection site, a reaction known as the hypersensitive response (HR). Xenobiotics could also induce HR-like lesions. The cellular defence responses involved in induced resistance are either activated directly or primed for augmented expression upon pathogen attack or xenobiotic exposure (Hulten et al., 2006). Many of the cell death regulators that have been characterized in humans, worms, and flies are absent from the Arabidopsis genome, indicating that plants probably use other regulators to control this process (Lam et al., 2001). The cell death response in plants is under strict genetic control, as evidenced by the existence of mutants that spontaneously form HR-like lesions (lesion-mimic mutants, At psi2 and Cat1AS) in many plant species, associated with the induction of other components of the plants’ defence arsenal, including accumulation of reactive oxygen species (ROS), expression of pathogenesis-related (PR) genes, production of phytoalexin, and the reinforcement of cell walls (Stone et al., 2000). In the presence of OTA, the growth of Arabidopsis thaliana on media was significantly inhibited; in addition, cell death was observed with features resembling the HR-type lesions in excised leaves that were infiltrated with this toxin. There was also evidence that cell death was induced by OTA, such as the occurrence of an oxidative burst and the deposition of callose and phenolic compounds (autofluorescence) (Peng et al., 2010).

Although the role of toxins as effectors of disease susceptibility has been well characterized, there is little knowledge about the mechanisms of general or basal resistance of plants to toxins. To understand more about the process by which OTA disturbs cellular signalling and regulation and modulates physiological signals, a genome-wide coverage method was used that allowed identification of a group of early regulated genes, including transcription factors, that are potentially involved in the transcriptional reprogramming observed during the later stages and in the regulation of the cell death process. By comparing transcript and protein patterns, protein expression driven directly by transcript abundance can be distinguished from that regulated post-transcriptionally.

Here, an expansive view of the early stage of OTA-induced cell death and the regulation of the toxicity response process in A. thaliana is reported from an integrated bioinformatics analysis of proteomic and transcriptomic data sets. It was found that (i) a number of xenobiotic and ROS-inducible genes were also up-regulated; (ii) antioxidant enzyme defence responses were enhanced; (iii) photosynthesis and photorespiration were compromised; and (iv) the biological membrane played a relatively large role in transport. Genes and proteins involved in important functional organs and key metabolic pathways are required for the regulation of cell death; therefore, this study brings new insights into the regulation of the toxicity response to OTA in Arabidopsis.

Materials and methods

Chemicals

OTA was extracted and purified as described previously (Peng et al., 2010). All other chemicals were of high purity grade.

Plant materials and OTA treatments

Arabidopsis Col-0 wild-type plants were germinated on Murashige and Skoog (MS) medium containing 2% sucrose and 0.8% Phytagar after a 3 d vernalization period at 4 °C under the following conditions: 16 h light/8 h dark period, photosynthetic photon flux density 100 μmol m−2 s−1, 22 °C, and 60% relative humidity. Seven-day-old seedlings were planted into soil. Four-week-old plants were used for the experiments.

Briefly, OTA (2 mM and 1 mM) was infiltrated into leaves using a syringe without a needle, as described previously (Gechev et al., 2004). Methanol-infiltrated plants served as controls. Excised leaves were incubated in Petri dishes containing OTA or the corresponding concentration of solvent (methanol) used as a control (Peng et al., 2010) under continuous light or dark at room temperature (22 °C). Samples were taken after 3, 8, 16, 24, 48, and 72 h of treatment.

Chlorophyll content measurement

Chlorophyll content was measured after extraction with hot ethanol, as described by Anderson and Rowan (1965). Leaf tissues (0.2 g fresh weight) were ground and then homogenized in 5 ml of acetone, and they were then placed in the dark for 1 h at room temperature. Extracts were filtered, and the liquid supernatants were measured by the UV-Vis method.

Measurement of the relative leakage rate

Cell death was also determined by electrolyte leakage from the leaves (i.e. the increased conductivity) according to the method of Peng et al. (2010). To avoid differences in the ion balance due to the treatment, the results were expressed as relative conductivity rather than absolute conductivity.
Transmission electron microscopy

For electron microscopy, the treated leaves were cut into 2 mm × 2 mm pieces and fixed in a 3% glutaraldehyde-0.1 M pH 7.2 phosphate-buffered saline (PBS) solution at room temperature for 3 h. Samples were post-fixed in 1% osmium tetroxide in 50 mM pH 7.2 sodium cacodylate, dehydrated in a graded ethanol series, and embedded in Spur’s resin. Blocks were cut with a diamond knife on an LKB-8000 Ultracut ultramicrotome to obtain ultrathin sections, which were stained with 2% uranyl acetate and lead citrate and then examined using a JEM-1230 transmission electron microscope (JEOL, Japan).

RNA extraction

Total RNAs were prepared using an RNAprep pure Plant kit (Tiangen Inc.) according to the manufacturer’s instructions. They were subsequently purified using an RNaseasy RNA purification kit (Qiagen, http://www.qiagen.com/) with on-column DNase digestion. Equal loading was verified by ethidium bromide staining of the gel.

Real-time RT-PCR

For quantitative RT-PCR analysis, leaves were infiltrated as above and samples were collected 3, 8, or 24 h later. Total RNA was isolated as above. The quality of the RNA was assessed with Lab-on-chip analysis using a 2100 Bioanalyzer (Agilent, http://www.agilent.com/). cDNA was synthesized using a first-strand cDNA Quantscript RT kit (Tiangen Inc.) according to the manufacturer’s instructions. Real-time PCR experiments were performed in triplicate in 25 μl volumes using RealMasterMix (SYBR green) (Tiangen Inc.) in an ABI 7500 Real-time PCR machine (Applied Biosystems, http://www.appliedbiosystems.com/). The thermal cycling program was set as follows: 50 °C for 10 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 25 s, 58–60 °C for 25 s (optimized for each primer pair), and 72 °C for 30 s, a melting curve stage at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, and 60 °C for 15 s. If non-specific fluorescence was observed in the melting curve, the reaction was excluded and repeated. Gene-specific primers (Supplementary Table S1 available at JXB online) were designed for each of the target genes and for the reference gene (Actin2). Samples from each of three biological replicates were assayed in triplicate. Expression values were normalized to those of Actin2 replicates were assayed in triplicate. Expression values were described previously (Xu et al., 2006; Nafisi et al., 2007).

ROS level and the lipid peroxidation status

The ROS content of the leaves’ response to OTA at 0.1, 0.25, and 1 mM for 24 h was measured as described previously (Peng et al., 2010) and the malondialdehyde (MDA) content was analysed as described previously (Xu et al., 2009).

Measurement of the photosynthetic activity

The photosynthetic activity (Pn) was detected using the LI-6400XT photosynthetic system (LI-COR, Lincoln, NE, USA). The LI-6400XT measures gas exchange over the same leaf area with full control of environmental variables. The concentration of CO₂ was set at 400 μmol mol⁻¹, photosynthetic photon flux density 100 μmol m⁻² s⁻¹, 22 °C, and 60% relative humidity.

Protein extraction and two-dimensional electrophoresis

The extraction of total proteins was performed as described by Chan et al. (2007) with some modifications. All procedures described below were carried out at 4 °C. Briefly, 1 g of leaves from 60 single plants were immersed in 70 ml of 0.25 mM OTA in several Petri dishes for 8 h. The extracted protein was solubilized in 500 μl of lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT; Sigma, http://www.sigma-aldrich.com/), 1% (v/v) pH 4–7 IPG buffer, 1% (v/v) pH 3–10 NL IPG buffer (GE Healthcare), and 0.5% (v/v) protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was quantified using a 2-D Quant kit (GE Healthcare) with bovine serum albumin as the standard. The protein samples were stored at −80 °C prior to use.

Two-dimensional electrophoresis (2-DE) of protein extracts was performed using a two-dimensional electrophoresis system (GE Healthcare, http://www.gehealthcare.com/), according to the manufacturer’s instructions. A 250 μl aliquot of total protein (400 μg) was loaded in 13 cm, pH 3–10 NL IPG strips (GE Healthcare) for isoelectric focusing. Prior to the electrophoresis in the second dimension, the IPG strips were equilibrated by reduction with β-mercaptoethanol and carboxymethylation with iodoacetamide. The equilibrated strips were run on 12.5% SDS-polyacrylamide gels at 10 mA per gel for 1 h and 20 mA per gel until the dye front (sealing the IPG Strip gel with agarose sealing solution, containing 0.5% bromophenol blue) reached the bottom end of the gel. Proteins were visualized with Coomassie Brilliant Blue R-250 after 1 h protein fixation in a solution of 50% ethanol, 10% acetic acid, and 40% water. The gels were destained with a solution of 30% ethanol, 8% acetic acid, and 62% water for 2 h and then washed five times with water. Image digitization was carried out with an Image Scanner (GE Healthcare) in transmission mode. Protein expression levels in 2D gel images were compared using the Image Master 2D Elite software (GE Healthcare). To account for experimental variation, at least three gels, resulting from protein extracts obtained from independent experiments, were analysed for each treatment. Statistical analysis of the data was performed using SPSS software, version 11.5 (SPSS Inc., Chicago, IL, USA). The normalized intensity of spots on three replicate 2D gels was averaged, and the standard deviation was calculated for each treatment. A two-tailed unpaired Student’s t-test was used to determine whether the relative change between control and OTA-treated samples was statistically significant (Chan et al., 2007). Only spots that changed significantly in averaged normalized spot volume were excised for protein identification.

Protein identification

Protein spots with significant changes (at least 2-fold) were carefully cut out from Coomassie Brilliant Blue R-250-stained gels and subjected to in-gel trypsin digestion according to Sun et al. (2007) with minor modifications. MALDI-TOF/TOF MS/MS (matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry) experiments were carried out according to Zhang et al. (2006) with minor modifications. GPS Explorer™ software, version 3.6 (Applied Biosystems) was used to create and search files with the MASCOT search program (Matrix Science, http://www.matrixscience.com/) for peptide and protein identification. The NCBI Greenplant Database 2009 was used for the search and was restricted to tryptic peptides. Carboxymethylation and oxidation were selected as variable modifications. One missed cleavage was allowed. Precursor error tolerance was set to ±0.2 Da, and MS/MS fragment error tolerance was set to ±0.3 Da. All of the proteins identified had protein scores >61 and individual ion scores >21, with expected P-values <0.05. All of the MS/MS spectra were further validated manually.

Digital gene expression: tag profiling (DGE)

Sequence tag preparation was performed with the Illumina Digital Gene Expression Tag Profiling Kit (Illumina, Inc.) according to the manufacturer’s protocol. In brief, 6 μg of total RNA was incubated with oligo(dT) magnetic beads to adsorb the polyadenylated RNA fraction. First- and second-strand cDNA was synthesized guided by oligo(dT), and bead-bound cDNA was subsequently digested with NlaIII to retain a cDNA fragment from
the most 3' CATG to the poly(A) tail. Subsequently, a GEX NlaIII adaptor (adaptor 1) containing a restriction site for MmeI was used to cut 17 bp downstream from the NlaIII site, thereby releasing 21–22 bp tags starting with the NlaIII recognition sequence, CATG. At this point, the fragments detached from the beads and, after dephosphorylation and phenol extraction, a second GEX adaptor (adaptor 2) was ligated at the site of MmeI cleavage. After 15 cycles of linear PCR amplification, 85 base strips were purified by 6% TBE PAGE. These strips were then digested, and the single-chain molecules were fixed onto a Solexa Sequencing Chip (flowcell) (Illumina, Inc., http://www.illumina.com/index.ilmn). Each molecule grew into a single-molecule cluster sequencing template through four types of nucleotides, labelled with four colours, were added, and sequencing by synthesis (SBS) was performed. Each tunnel generated millions of raw reads with a sequencing length of 35 bp. The raw sequences had 3' adaptor fragments as well as a few low-quality sequences and several types of impurities. The raw sequences were transformed into 17 bp clean tags, and tag counting was carried out using the Illumina Pipeline.

A pre-processed database of all possible CATG+17 nucleotide tags was created using reference gene sequences (ftp://ftp.arabidopsis.org/Sequences/blast_databases/TAIR9_blastsets/). All clean tags were mapped to the reference sequences, and no more than one nucleotide mismatch was allowed. The clean tags mapped to reference sequences from multiple genes were filtered. The remaining clean tags were designed as unambiguous clean tags. The number of unambiguous clean tags for each gene was calculated and then normalized to the number of transcripts per million clean tags (TPM) using the method described by Hoen et al. (2008) and Morrissy et al. (2009). Finally, a rigorous algorithm developed by the Beijing Genomics Institute (BGI) referring to ‘the significance of digital gene expression profiles [false discovery rate (FDR) < 0.001] (Audic and Claverie, 1997) was used to identify differentially expressed genes between two samples (Benjamini and Yekutieli, 2001), and absolute value of log2ratio >=1 (minimum of 2-fold difference) as the threshold to judge the significance of gene expression difference.

Statistical analysis
All statistical analyses were performed using Excel 2007 software and SPSS software, version 11.5. The results were considered to be statistically significant at $P < 0.05$. When the analysis was statistically significant, Duncan’s multiple range test was applied to the separate mean values.

Results

OTA induces lesion formation in Arabidopsis leaves

When an OTA solution (2 mM, 1 mM, or control) was infiltrated into leaves of 4-week-old Arabidopsis plants grown in soil in a greenhouse, macroscopic lesions formed on the infiltrated leaves within 1–2 d (Fig. 1A). To determine whether the response of A. thaliana to OTA is similar to its response to FB1 and AAL toxin, which both elicit necrotic lesion formation in detached tomato leaves (Stone et al., 2000; Gechev et al., 2004), detached leaves were incubated in Petri dishes containing 0.1 mM, 0.25 mM, or 1 mM OTA. As illustrated in Fig. 1A, macroscopic lesions formed on the leaves exposed to OTA for 3 d. Lesion formation was dose dependent and was evident at concentrations of >0.1 mM OTA. Surrounding the area of necrotic tissue, progressive yellowing of the leaf was clearly visible in the 1 mM and 0.25 mM OTA treatments (Fig. 1B). This yellowing is reflected in a loss of average chlorophyll content as the diameter of the necrotic lesion area increases (Fig. 1C).

Light is required for lesion formation in response to various pathogens (Guo et al., 1995) as well as in some lesion-mimic mutants and in transgenic plants that form spontaneous HR-like lesions (Genoud et al., 1998). As with FB1 (Stone et al., 2000), light is also required for lesion formation in response to OTA. As shown in Fig. 1B, OTA-elicited lesion formation in Arabidopsis leaves was greatly reduced in the dark.

The measurement of relative ion leakage was used as an indicator of the extent of cell death; the relative leakage rate in the OTA treatment group was always significantly higher than that in the control group, and the relative leakage rate in the light was higher than that in darkness (Fig. 1D). This showed that leaves of A. thaliana were sensitive to OTA, and light could accelerate the process of cell death.

Ultrastructural changes during OTA exposure

Ultrastructural examination of Arabidopsis leaves floating in 0.25 mM OTA under continuous light at room temperature (22 °C) revealed very few differences between OTA-treated and methanol-treated controls at 3 h (Fig. 2G), and the cytological damage induced by OTA was first observed at 8 h, prior to chlorophyll reduction. At 8 h, the separation of the plasma membrane from the cell wall, fold formation, chromatin condensation, and the margination and breaking of the nucleolus were observed (Fig. 2A–C). At 24 h, the deformation of cell organelles became more severe, the structures of the mitochondria and chloroplasts were destroyed, the mitochondrial matrix escaped out of the mitochondria, the thickness of the membrane was uneven, part of the nuclear membrane had become invaginated, and some of the nuclei were distorted (Fig. 2D–F). The appearance of control leaves showed changes at 4 d (not shown); however, the obvious destruction of the organelles could be observed at 3 d at the ultrastructural level. Since the condensation of nuclei is an important morphological trait characteristic of apoptosis, the fact that these symptoms were observed in 0.25 mM OTA-treated leaves at 8 h demonstrated that OTA induced cell death in Arabidopsis leaves.

Potentiation of gene expression during OTA exposure

To verify further that a defence response took place and induced cell death under the treatment conditions, 4-week-old Arabidopsis leaves were treated with 0.25 mM OTA for 3, 8, and 24 h, and control leaves were treated with 0.122% methanol. Gene expression ratios relative to the control treatment are shown in Fig. 3. Several genes induced by OTA were characterized by potentiated expression of the salicylic acid-inducible marker gene PR1 and aminocyclopropane carboxylate synthase ACS6. These genes were up-regulated as the treatment time increased. The expression of respiratory burst oxidase homologue C (AtbohC) and AtbohD increased dramatically after 3 h and 24 h, respectively.
The expression of the APX antioxidant gene was in accordance with the ROS level (Fig. 4A). There was a continuous increase in the ROS content with the increase of OTA concentration. The application of OTA to excised A. thaliana leaves significantly accelerated the increase in MDA (Fig. 4B).

Proteomic analysis of OTA-induced cell death

Protein samples for 2-DE were obtained from detached leaves after treatment with OTA (Fig. 5). Changes in the abundance of proteins were measured and compared between control and OTA-treated samples in three independent replicates. Analysis of the 2-DE pattern revealed that most protein spots on the gel had an acidic pI value in the range of pH 4–7 and a molecular mass between 15 kDa and 80 kDa. Approximately 1032–1061 protein spots could be detected on 2D gels after ignoring very faint spots and spots with undefined shapes and areas. Quantitative analysis of spot intensity by integration of the staining signal for each gel and image analysis revealed that the levels of 27 of the resolved proteins changed in an OTA-dependent manner (ratio >1.5) in three independent experiments. The full data set is available in Supplementary Table S2 at JXB online. The spots selected for further analysis are indicated in Fig. 5. Using MALDI-TOF/TOF MS/MS, the 27 protein spots that showed relatively high abundance were analysed. The results of this analysis are summarized...
in Table 1. Four of these differentially expressed proteins (SPs 4, 10, 25, and 27) did not show a total ion score, and the remaining 23 were submitted to the MASCOT search engine for database searching. Among these 23 protein spots, 18, representing 13 different proteins, were identified with significant Mascot scores \( P < 0.05 \), whereas five protein spots (SPs 2, 9, 17, 18, and 19) showed relatively low total ion scores.

It is noteworthy that six protein spots (SPs 5, 8, 22, 23, 24, and 26) were identified as the same protein, ribulose bisphosphate carboxylase (Rubisco) (Table 1). The location of these spots in the gels differed in molecular mass and pI (Fig. 5), indicating that they might be catabolites of these spots in the gels differed in molecular mass and pI. The large subunit and small chain of Rubisco were also up-regulated. In addition, seven proteins were identified as enzymes involved in basic metabolism, including transketolase (SP 1), serine hydroxymethyltransferase (SHM1, SP 3), aminomethyltransferase (SP 6), phosphoribulokinase (PRK, SP 11), sedoheptulose-bisphosphatase (SBPASE, SPs 12), fructose-bisphosphate aldolase (SP 14), and phosphoglycerate kinase (PGK, SP 20). The three transferases (SPs 1, 3, and 6) were all down-regulated, indicating that basic cellular metabolism decreased. PRK, SBPASE, and fructose-bisphosphate aldolase were all up-regulated. These enzymes of the glycolytic pathway and the Calvin cycle catalyse the production of ATP upon exposure to OTA. In addition, a putative RNA-binding protein \( g5bf \) (SP 7, 1.6-fold down-regulated), an annexin (AnnAt1, SP 13, 1.8-fold up-regulated), and four photosynthesis-related proteins (SPs 15, 16, 20, and 21) were identified. Although the function of the putative RNA-binding protein is unknown, \( g5bf \) was also shown to be differentially expressed in R17 cucumber induced by powdery mildew fungus (Fan et al., 2009); it has been correlated with mycotoxin and fungi stresses.

**Transcriptional analysis of OTA-induced cell death**

Because changes at the transcript level are not necessarily reflected at the protein level, and to obtain more information about the nature of OTA-triggered cell death, a DGE tag profiling analysis approach was pursued to quantify gene changes in response to OTA. This strategy was based on Illumina high-throughput sequencing technology and the newly assembled Arabidopsis reference genome. Samples for DGE analysis were collected 8 h after incubation with 0.25 mM OTA, and the control was treated with 0.122% methanol. The Arabidopsis reference genome contains 33 518 genes, and 30 856 (92.06%) of these genes have CATG sites. At the 8 h time point, 197 genes were up- or down-regulated by at least 5-fold, and 3118 (10.11%) genes were up- or down-regulated by at least 2-fold. Of these genes, 1923 showed increased expression, demonstrating that a massive transcriptional reprogramming took place in the OTA treatment samples compared with the methanol-treated controls. To verify the DGE data, selected genes, including \( APX \), \( PRI \), and \( ACS6 \) from the DGE analyses, were detected by real-time PCR analysis. Quantification of the signals showed that all the patterns of gene expression were consistent with the DGE results, although the ratios varied to some extent (Fig. 3).

The Gene Ontology (GO) classificiations of the most highly regulated genes are shown in Fig. 6. According to the putative homology to sequences present in public databases, the differentially expressed genes were classified into 14 different cellular component categories. Through GO analysis, 164, 337, 519, and 449 genes were found to be differentially expressed in mitochondria (5.3%), the cell nucleus (10.8%), chloroplasts (16.6%), and the plasma membrane (14.4%), respectively. Genes located in these organelles were pivotal and necessary in response to stress, providing defence against mycotoxins. Table 2 shows the genes that were most regulated at each time point and their classification into functional categories including antioxidant metabolism, detoxification of xenobiotics, resistance...
and defence, mitogen-activated protein kinase (MAPK) signalling, transcription factor, hormone signalling, transport, photosynthesis, and the proteasome pathway. The full data set is available in Supplementary Table S3 at JXB online.

The DGE data indicated that genes involved in the detoxification of xenobiotics, cytochrome P450 family proteins (including CYP81D8 (1899), CYP71A12 (890), CYP71A22 (504), and CYP81D1 (202)), UDP-glucuronosyltransferases (UDPGT) (including UGT73B4 (8042), UGT74F1 (347), UGT88A1 (18.64), UGT75D1 (13.83), and UGT75B1 (20.72)), as well as ATP-binding cassette (ABC) transporter family members, were dramatically up-regulated. Cytochrome P450 is essential for the primary or phase I metabolism of lipophilic xenobiotics. OTA-inducible Arabidopsis cytochrome P450s are homologues of mammalian cytochrome CYP. Biotransformation of many xenobiotics involves UDPGT and GST, which catalyse conjugation reactions (phase II enzymes). OTA is hydrophobic, but conjugates with minor toxicity and high water solubility could be recognized by a glutathione pump, such as ABC and the ABC transporter, and be transported to the vacuole across the membrane (Taysse et al., 1998). Recently, under certain circumstances, P450s have been shown to produce ROS, resulting in oxidative stress and cell death (Gonzalez, 2005). Xenobiotics were detoxified on a large scale before peak oxidative stress in response to OTA treatment.

Genes such as FSD1 (2.11), CAT1 (8.06), TPX2 (10.68), GPX6 (4.21), PRXIIF (4.25), GPX2 (3.17), GPX3 (2.32), PER50 (3.85), PER12 (2.05), APX3 (4.49), MDAR2 (2.79), and MDAR1 (2.54) are up-regulated. These well-known antioxidant genes are considered to be early markers of oxidative stress.

Several regulatory genes, including the transcription factors WRKY 75 (5876), AtbZIP15 (445), AP2 domain-containing transcription factor (2522), ANAC 042 (534), ATHB 8 (504), WRKY 6 (34.52), ATMYB102 (621), and ERF11 (12.48), were up-regulated with OTA treatment. A diverse group of transcription factors are induced early, during the first 8 h of treatment with 0.25 mM OTA: two WRKY family proteins, a zinc finger protein, an AP2 domain protein, a MYB family member, two NAC domain proteins, and an ERF domain protein are up-regulated. On the other hand, MAPKKK 19 (504) was the most regulated gene during the MAPK cascade activation process. Activation of
the MAPK cascade by treatment with OTA was able to
dramatically up-regulated the expression of
WRKY75 (5786), WRKY6 (34), and WRKY18 (4.8). The changes
in the MAPK cascade and transcription factors amplify the
signal promptly, protecting the plants against OTA and
oxidative stress.

Up-regulation of several elements of the ubiquitin–
proteasome pathway, among them the ubiquitin-conjugating
enzymes UBC3 (39.96) and UBC16 (12.58), the UBC13–
MMS2 complex (6.15), and many genes from the regulatory
and catalytic subunits of the proteasome (Table 2), was
observed. The ubiquitin–proteasome system (UPS) targets
numerous intracellular regulators. The UPS is not only
involved in the degradation of short-lived, damaged, and
misfolded proteins in the cytosol and nucleus during stress
and cell death responses, but it also interacts with other
components of the cell death machinery, most notably
caspases in animal cells or caspase-like proteins in plant cells.
In particular, the stress factor UBC3 participates in all stress
responses, playing an important regulatory role (Richard
and Vierstra, 2009; Santner and Estelle, 2010).

The pathogenesis-related genes PR5 (11.1) and PRI
(17.2), plant defensin PDF1.2¢ (119), and three universal
stress proteins (USPs) were strongly up-regulated at
the mRNA level during OTA-induced cell death. Repression
of the majority of regulated genes and overexpression of two
pivotal ethylene biosynthesis genes, ACS2 and ACS6, were
also observed (Table 2). The senescence-related gene SRG2
(71.5) and some senescence-activated genes, including
SAG13 (4.92), SAG18 (2.40), and SAG21 (3.41), were
overexpressed. Most of the genes implicated in auxin
responses to OTA were negatively regulated, just as H2O2
can also negatively regulate the auxin responses through
activation of the MAPK cascade in Arabidopsis. ROS can
increase ethylene production through the activation of ACC
(1-aminocyclopropane-1-carboxylate) synthase and tran-
scriptional up-regulation of ACC oxidase, two enzymes
involved in ethylene biosynthesis. In turn, ethylene can
greatly potentiate the oxidative burst (Gechev et al., 2004).
In contrast to ROS and ethylene, there was no indication of
accumulation of the plant hormones jasmonic acid (JA) and
gibberellic acid (GA). Two JA signalling genes, THI2.2
(−2.75) and VSP1 (−3.35), were repressed. The thionin
genes, arguably the best markers of the presence of JA,
were down-regulated. GA2 oxidase 6 (3.45) was up-
regulated, but a putative GA-responsive protein (−3.21)
was down-regulated, whereas several GA2 oxidases that can
degradate GA were up-regulated. Plant hormone signalling
is also regulated by ubiquitylation, and the UPS plays an
essential role in hormone perception and response (Santner
and Estelle, 2010). These signals interact to determine the
ultimate fate of the plant cell.

**Discussion**

Changes in mRNA levels do not always lead to similar
alterations in protein levels or enzyme activities. Neverthe-
less, a comprehensive transcriptome and proteome analysis
gives an impression that the dynamics of the cellular
processes are involved in the cell death machinery in
response to OTA

**Photosynthesis and photorespiration**

Most of the photosynthesis-related genes that were identi-
fied in this study were down-regulated or repressed. Several
proteins from both the light reactions (oxygen-evolving
enhancer and chlorophyll a/b-binding proteins) and dark
reactions (Rubisco and Rubisco subunit-binding proteins)
of photosynthesis were detected in the proteomic analysis in
response to OTA (Fig. 7, Table 1). Photosynthesis provides
oxygen and increases the energy state during the switch to defence and regulation of biosynthetic activities. SP 16, chlorophyll a/b-binding protein, was down-regulated (–1.9), coinciding with the gene CAB identified in DGE (Fig. 5, Table 2). The essential adjustment factor CRN1 in the chlorophyll degradation process increased strongly (Fig. 5), and chlorophyllase ATCLH1 (–5.07) decreased, consistent with the slight chlorosis in leaves and the loss of average chlorophyll content (Fig. 1). The up-regulation of the Rubisco large subunit and small chain indicated that Rubisco was degraded, consistent with reports that Rubisco degradation can be activated by ROS (Liu et al., 2008). The negative effects of OTA on the large subunits of the three different Rubisco spots (Table 1) revealed that the decrease in Rubisco activity can be explained, in part, by lower Rubisco availability. Photosynthetic activity was reduced (Fig. 4C), which could be associated with the loss of average chlorophyll content and the decrease in Rubisco activity in response to OTA.

Meanwhile, the SHM1 gene was suppressed, coinciding with its protein expression (Fig. 7, Table 1). SHM1 encodes the mitochondrial isoform of serine hydroxymethyltransferase (SHMT), which, combined with glycine decarboxylase, catalyses an essential sequence of the C2 cycle, namely the
conversion of two molecules of glycine into one molecule each of CO$_2$, NH$_4^+$, and serine. Photorespiration is caused by the dual affinity of Rubisco for both CO$_2$ and molecular oxygen, and this cycle involves three organelles (chloroplasts, peroxisomes, and mitochondria) (Voll et al., 2006). Rubisco degradation and loss of SHMT in Arabidopsis result in a compromised photorespiratory C$_2$ cycle and overproduction of ROS, which makes the sample more susceptible to OTA stress (Moreno et al., 2005). This finding could explain why OTA-elicited lesion formation in Arabidopsis leaves was greatly reduced in the dark.

Two kinases related to photosynthesis, PRK (1.6) and PGK (–1.7), participate in photosynthetic carbon dioxide fixation. PRK can catalyse reactions by binding ATP and active Rubisco. PRK was up-regulated, but its gene expression was suppressed at the mRNA level 8 h after OTA treatment. The PGK protein and mRNA levels appeared to be correlated in terms of their expression trends. Oxygen-evolving

| Spot | Ratio | NCBI accession no. (gi) | Protein name | Mol. wt (kDa) theor./exp. | pI theor./exp. | Mascot score/threshold | Total ion score | NP/ PD | Percentage sequence coverage |
|------|-------|-------------------------|--------------|--------------------------|---------------|------------------------|----------------|-------|-------------------------------|
| 1    | –2.5  | gi|73290685 Transketolase, putative (Arabidopsis thaliana) | 81.9/80 | 5.8/5.5 | 383/46 | 256 | 5/24 | 13 |
| 3    | –1.8  | gi|5235745 SHM1 (serine hydroxymethyltransferase 1) (Arabidopsis thaliana) | 57.4/55 | 8.1/7.8 | 266/45 | 99 | 7/25 | 17 |
| 5    | 1.8   | gi|94432 Ribulose bisphosphate carboxylase (Arabidopsis thaliana) | 47.6/46 | 6.1/6.6 | 256/4/7 | 128 | 4/20 | 19 |
| 6    | –3.7  | gi|5221119 Aminomethyltransferase, putative (Arabidopsis thaliana) | 44.7/45 | 8.5/8.0 | 469/44 | 350 | 8/19 | 28 |
| 7    | –1.6  | gi|850021 Putative RNA-binding protein (Arabidopsis thaliana) | 42.1/40 | 7.7/7.5 | 452/46 | 358 | 7/16 | 21 |
| 8    | 1.7   | gi|94432 Ribulose bisphosphate carboxylase (Arabidopsis thaliana) | 47.6/46 | 6.1/7.2 | 382 | 188 | 17/24 | 22 |
| 11   | 1.6   | gi|522551 PRK (phosphoribulokinase) (Arabidopsis thaliana) | 44.4/44 | 5.7/5.1 | 342/46 | 236 | 7/17 | 19 |
| 12   | 1.9   | gi|5228194 SBPASE (sedoheptulose-bisphosphatase) (Arabidopsis thaliana) | 42.4/43 | 6.1/4.8 | 519/47 | 427 | 10/15 | 27 |
| 13   | 1.8   | gi|429207 AnnAt1 (annexin Arabidopsis 1) (Arabidopsis thaliana) | 35.7/35 | 5.19/5.2 | 232/46 | 73 | 2/20 | 10 |
| 14   | 1.7   | gi|5236768 Fructose-bisphosphate aldolase, putative (Arabidopsis thaliana) | 38.3/38 | 5.65/5.8 | 205/46 | 110 | 5/14 | 20 |
| 15   | 1.5   | gi|403751 Plastid-lipid-associated protein PAP (Arabidopsis thaliana) | 30.4/30 | 5.82/4.8 | 204/46 | 141 | 3/10 | 13 |
| 16   | –1.9  | gi|6374 Chlorophyll a/b binding protein (LHCP AB 180) (Arabidopsis thaliana) | 25.0/29 | 5.12/4.9 | 132/45 | 75 | 2/9 | 23 |
| 20   | –1.7  | gi|022805 PGK (phosphoglycerate kinase) (Arabidopsis thaliana) | 41.9/28 | 4.9/3.7 | 82/46 | 40 | 2/7 | 9 |
| 21   | 1.6   | gi|5222166 PBSP-1 (oxygen-evolving enhancer protein 2) (Arabidopsis thaliana) | 28.1/26 | 6.9/5.2 | 216/46 | 150 | 4/11 | 25 |
| 22   | 1.5   | gi|7525041 Ribulose bisphosphate carboxylase large subunit (Arabidopsis thaliana) | 52.9/18 | 5.88/4.7 | 316 | 237 | 5/15 | 11 |
| 23   | –1.6  | gi|54300670 Ribulose bisphosphate carboxylase large subunit (Neostenanthera myristicifolia) | 52.5/20 | 6.14/5.5 | 269/47 | 205 | 3/14 | 7 |
| 24   | –1.8  | gi|2155831 Rubisco large subunit (Prostanthera nivea) | 28.2/20 | 8.8/4.6 | 447/46 | 388 | 6/11 | 27 |
| 26   | 1.6   | gi|3926229 F1O19.10/F1O19.10 (Arabidopsis thaliana) | 14.7/14 | 5.69/5.2 | 385/47 | 307 | 7/10 | 52 |

Table 1. Identification of intracellular proteins showing differential expression under OTA stress using MS/MS analysis. Spot numbers correspond to those in Fig. 3. Ratio is the average change in abundance expressed as mean intensity ±SD from three independent treatments; Protein name, matched protein description and the species of the matched protein; NCBI accession no., accession number from the NCBI database of matched proteins; Theo. mol. wt (kDa)/pI, the theoretical molecular mass and isoelectric point based on the amino acid sequence of the identified protein; Exp. mol. wt (kDa)/pI, experimental molecular mass and isoelectric point estimated from the 2D gels; Mascot score/threshold, score obtained from MASCOT for each match and amino acid sequence coverage for the identified proteins; Total ion score, score obtained from MASCOT for all matches; NP, the number of matched peptides; PD, peptides detected.
enhancer protein 2 of photosystem II (PSII) subunit P PsbP-1 (3.5) was up-regulated at the protein level, but it was simultaneously repressed at the mRNA level. However, PsbP-1 was overexpressed at the mRNA level 3 h after OTA treatment. The protein level was higher, due to the protein accumulation at the early stage (Fig. 7). In addition, the oxygen-evolving complex (OEC; containing PsbO, PsbP, and PsbQ) is localized on the lumenal side of PSII, responsible for energy transfer and charge separation.
### Table 2. Global changes in gene expression during OTA-induced cell death

| Gene category                        | Gene       | Gene description                           | Fold change (log$_2$) | ID          |
|--------------------------------------|------------|--------------------------------------------|-----------------------|-------------|
| **Antioxidant metabolism**           | FSD1       | Fe superoxide dismutase                     | 2.11 (1.08)           | AT4G25100   |
|                                      | CAT1       | Catalase                                    | 8.06 (3.00)           | AT1G20630   |
|                                      | APX3       | Ascorbate peroxidase 3                      | 4.49 (2.17)           | AT4G35500   |
|                                      | SAPX       | Stromal ascorbate peroxidase                | 2.16 (1.11)           | AT4G08390   |
|                                      |            | Anionic peroxidase, putative                | 73.12 (6.19)          | AT1G14540   |
|                                      |            | Peroxidase, putative                        | 16.93 (4.08)          | AT5G39580   |
|                                      | TPX2       | Thioredoxin-dependent peroxidase 2          | 10.68 (3.42)          | AT1G65970   |
|                                      |            | Peroxidase, putative                        | 4.40 (2.14)           | AT4G37530   |
|                                      | CM1        | Chorismate mutase 1                         | 4.37 (2.13)           | AT3G29200   |
|                                      | PXO01F     | Peroxiredoxin IIF                          | 4.25 (2.09)           | AT3G06050   |
|                                      | GPX6       | Glutathione peroxidase 6                    | 4.21 (2.08)           | AT4G11600   |
|                                      | PER50      | Peroxidase 50                               | 3.85 (1.94)           | AT4G37520   |
|                                      |            | Glutathione peroxidase, putative            | 3.39 (1.76)           | AT1G63460   |
|                                      | GPX2       | Glutathione peroxidase 2                    | 3.17 (1.67)           | AT2G31570   |
|                                      | GPX3       | Glutathione peroxidase 3                    | 2.32 (1.21)           | AT2G43350   |
|                                      | PER12      | Peroxidase 12                               | 2.05 (1.03)           | AT1G71695   |
|                                      | MDAR2      | Monodehydroascorbate reductase (NADH)       | 2.79 (1.48)           | AT5G03630   |
|                                      | MDAR1      | Monodehydroascorbate reductase, putative    | 2.54 (1.35)           | AT3G02880   |
|                                      | AOX1A      | Alternative oxidase                         | 4.51 (2.17)           | AT3G22370   |
|                                      | AOX1D      | Alternative oxidase                         | 96 (4.59)             | AT1G32350   |
| **Detoxification of xenobiotics**    | ATGSTU25   | Glutathione S-transferase                   | 3383 (11.72)          | AT1G17180   |
|                                      | ATGSTU2    | Glutathione S-transferase tau 2             | 1632 (10.67)          | AT2G29490   |
|                                      | ATGSTU9    | Glutathione S-transferase                   | 1573 (10.62)          | AT5G62480   |
|                                      | ATGSTU10   | Glutathione S-transferase                   | 594 (9.21)            | AT1G74590   |
|                                      | ATGSTU8    | Glutathione S-transferase                   | 564 (9.14)            | AT3G09270   |
|                                      | ATGSTU12   | Glutathione S-transferase                   | 415 (8.69)            | AT1G69920   |
|                                      | ATGSTU11   | Glutathione S-transferase                   | 226 (7.82)            | AT1G69930   |
|                                      | ATGSTU1    | Glutathione S-transferase                   | 152 (7.24)            | AT2G29490   |
|                                      | CYP81D8    | Cytochrome P450, family 81, subfamily D, polypeptide 8 | 1899 (10.89)       | AT4G37370   |
|                                      | CYP71A12   | Cytochrome P450, family 71, subfamily A, polypeptide 12 | 890 (9.70)       | AT2G30750   |
|                                      | CYP71A22   | Cytochrome P450, family 71, subfamily A, polypeptide 22 | 504 (8.98)       | AT3G48310   |
|                                      | CYP81D1    | Cytochrome P450, family 81, subfamily D, polypeptide 1 | 202 (7.66)       | AT3G28740   |
|                                      | UGT73B4    | UDP-glycosyltransferase 73B4                | 8042 (12.97)          | AT2G15490   |
|                                      | UGT74F1    | UDP-glycosyltransferase                     | 347 (8.44)            | AT1G05680   |
|                                      | UGT88A1    | UDP-glycosyltransferase                     | 16.64 (4.22)          | AT2G30140   |
|                                      | UGT75D1    | UDP-glycosyltransferase                     | 13.83 (3.79)          | AT4G15550   |
|                                      | UGT75B1    | UDP-glycosyltransferase                     | 20.72 (4.37)          | AT1G05560   |
|                                      | UGT85A1    | UDP-glycosyltransferase                     | 13.43 (3.75)          | AT1G22400   |
|                                      | ADH1       | Alcohol dehydrogenase 1                     | 2.81 (1.49)           | AT1G77120   |
|                                      | ADH        | Alcohol dehydrogenase, putative             | −2.55 (−1.35)         | AT2G22430   |
|                                      | MDR13      | ABC transporter family protein               | 3.1 (1.62)            | AT1G71960   |
|                                      |            | ABC transporter family protein               | 2.6 (1.40)            | AT1G54350   |
|                                      | ATM1       | ABC transporter of the mitochondrion        | −4.19 (−2.07)         | AT4G28630   |
|                                      |            | ABC transporter of the mitochondrion        | −3.95 (−1.98)         | AT5G06530   |
|                                      |            | ABC transporter of the mitochondrion        | −3.87 (−1.95)         | AT2G31610   |
|                                      |            | ABC transporter of the mitochondrion        | −2.07 (−1.04)         | AT2G01320   |
| **Resistance**                       | PR5        | Pathogenesis-related gene 5                 | 11.1 (3.48)           | AT2G34350   |
|                                      | PR1        | Pathogenesis-related gene 1                 | 17.2 (4.11)           | AT2G14610   |
|                                      | PDF1.2c    | Plant defensin 1.2C                         | 119 (6.89)            | AT5G44440   |
|                                      | USP        | Universal stress protein                    | 5.39 (2.43)           | AT3G02840   |
|                                      |            | Universal stress protein                    | 7.47 (2.90)           | AT3G062550   |
|                                      |            | Universal stress protein                    | 4.06 (2.02)           | AT3G11930   |
|                                      |            | Universal stress protein                    | 4.48 (2.16)           | AT2G47710   |
|                                      | PLP2       | Phospholipase 2                             | 9.19 (3.2)            | AT2G26560   |
| Gene category       | Gene          | Gene description                              | Fold change (log2) | ID           |
|--------------------|---------------|-----------------------------------------------|--------------------|--------------|
| TSA1               | Tryptophan synthease | 3.96 (1.99)                                   | AT3G54640          |
| MAPK signalling    | MAPKKK1       | MAP kinase kinase kinase 1                    | 2.97 (1.57)        | AT4G08500    |
|                   | MAPKKK19      | MAP kinase kinase kinase 19                   | 504 (8.98)         | AT5G67080    |
|                   | MAPKKK5       | MAP kinase kinase kinase 5                    | 3.6 (1.8)          | AT5G66850    |
|                   | MAPKKK10      | MAP kinase kinase kinase 10                   | 16.16 (4.01)       | AT4G08470    |
|                   | MAPKK21       | MAP kinase kinase kinase 21                   | 11.92 (5.38)       | AT4G36950    |
| Transcription factor| WRKY75        | WRKY75 transcription factor                   | 5876 (12.52)       | AT5G13080    |
|                   |               | AP2 domain-containing transcription factor, putative | 2522 (11.30)       | AT1G71520    |
|                   |               | AP2 domain-containing transcription factor family protein | 742 (9.54)         | AT2G33710    |
|                   | ANAC042       | Arabidopsis NAC domain-containing protein 42  | 534 (9.06)         | AT2G43000    |
|                   | ANAC019       | Arabidopsis NAC domain-containing protein 19  | 10.70 (3.42)       | AT1G52890    |
|                   | ATHB8         | Homeobox gene 8                               | 504 (8.97)         | AT4G32880    |
|                   | AbZIP15       | bZIP transcription factor family protein      | 445 (8.80)         | AT2G35530    |
|                   | WRKY6         | WRKY6 transcription factor                    | 34.52 (5.11)       | AT1G62300    |
|                   | WRKY18        | WRKY18 transcription factor                   | 4.82 (2.27)        | AT4G31800    |
|                   | ATERF         | Cooperatively regulated by ethylene and jasmonate 1 | 15.9 (3.99)       | AT3G50260    |
|                   | ATMVB102      | Arabidopsis MYB-like 102                      | 621 (9.28)         | AT4G21440    |
|                   | PIF4          | Phytochrome-interacting factor 4              | -2.01(–1.00)       | AT2G43010    |
|                   | ERF11         | ERF domain protein 11, transcription factor   | 12.48 (3.64)       | AT1G28370    |
|                   | SZF1          | Transcription factor                          | 3.26 (1.71)        | AT3G55980    |
|                   | RAV2          | Transcription factor                          | 4.13 (2.04)        | AT1G68840    |
|                   | RHL41         | Transcription factor                          | 8.19 (3.03)        | AT5G59820    |
| Ageing             | SAG13         | Senescence-activated gene                     | 4.92 (2.30)        | AT2G29350    |
|                   | SAG21         | Senescence-activated gene                     | 3.41 (1.77)        | AT4G02380    |
|                   | SAG18         | Senescence-activated gene                     | 2.40 (1.26)        | AT1G71190    |
|                   | SRG2          | Senescence-related gene                       | 71.54 (6.18)       | AT3G60140    |
|                   | SRG3          | Senescence-related gene                       | 4.32 (2.11)        | AT3G02040    |
|                   | SRG1          | Senescence-related gene                       | 3.71 (1.89)        | AT1G17020    |
| Ethylene biosynthesis| ACS2       | 1-Aminocyclopropane-1-carboxylate synthase | 5.76 (2.52)        | AT1G01480    |
|                   | ACS6          | 1-Aminocyclopropane-1-carboxylate synthase 6  | 4.53 (2.18)        | AT4G11280    |
| Jasmionic acid     | THI2.2        | Thionin 2.2                                   | -2.75(–1.46)       | AT5G36910    |
|                   |               | Vegetative storage protein-like               | -2.20(–1.14)       | AT5G44020    |
| Gibberellic acid   | GA2OX6        | Gibberelin 2-oxidase 6                        | 3.45 (1.79)        | AT1G24200    |
|                   |               | Gibberelin-responsive protein, putative       | -3.21(–1.68)       | AT1G22690    |
| Auxin and responses| AXR3         | Auxin-resistant 3                             | -6.43(–2.68)       | AT1G04250    |
|                   |               | Auxin-responsive family protein               | -5.65(–2.50)       | AT1G66150    |
|                   | ARF19         | Auxin-responsive factor 19                    | -3.77(–1.92)       | AT1G19220    |
|                   | AUX1          | Auxin-resistant 1                             | -3.74(–1.91)       | AT2G38120    |
|                   | PIN7          | Auxin efflux transmembrane transporter        | -3.72(–1.90)       | AT1G23080    |
| Photosynthesis     | PSAG          | Photosystem I subunit G                       | 2.83 (1.50)        | AT1G55670    |
|                   | LHCA1         | Chlorophyll binding                           | 5.64 (2.49)        | AT3G54890    |
|                   | LHB1B1        | Chlorophyll binding                           | 4.99 (–2.3)        | AT2G34430    |
|                   | LHCb5         | Light-harvesting complex of photosystem II 5 | -2.14(–1.1)        | AT4G10340    |
|                   | LHCb4.2       | Light-harvesting complex PSII                 | 2.11 (1.08)        | AT3G08940    |
|                   | CAB1          | Chlorophyll a/b-binding protein 1             | -2.62(–1.38)       | AT1G29930    |
|                   | CAB3          | Chlorophyll a/b-binding protein 3             | -2.68(–1.42)       | AT1G29910    |
|                   | PRK           | Phosphoribulokinase                           | -2.43(–1.28)       | AT1G32060    |
|                   | ATCLH1        | Chlorophyllase                                | -5.07(–2.34)       | AT1G19670    |
|                   | PSB28         | Photosystem II reaction centre PSB28 protein | -2.87(–1.52)       | AT4G28660    |
|                   | PabQ          | Oxygen-evolving enhancer 3 (PabQ)             | -2.34(–1.22)       | AT1G14150    |
|                   | PSB2N         | Photosystem II subunit T                      | 3.02 (1.59)        | AT3G21055    |
|                   | PSAD-1        | Photosystem I subunit D-1                     | -2.14(–1.10)       | AT4G02770    |
|                   | PSBO-1        | PSII oxygen-evolving complex I                | -2.05(–1.03)       | AT5G66570    |
|                   | PETF1         | Plastocyanin 1                                | -2.46(–1.29)       | AT1G76100    |
| Transporter       | TIP2          | Tonoplast intrinsic protein 2                 | -2.74(–1.45)       | AT3G26520    |
|                   | PIP1B         | Plasma membrane intrinsic protein 1B          | -2.12(–1.08)       | AT2G45960    |
for photosynthetic oxygen evolution; thus, PSII is a target within the photosynthetic apparatus for both biotic and abiotic stress conditions (Pérez-Bueno et al., 2004; Shinya et al., 2010). Previous research showed that Tobamovirus infection induced an inhibition of PSII electron transport, disturbing the OEC; the levels of the PsbP and PsbQ extrinsic proteins were lowered to different extents (Pérez-Bueno et al., 2004). In the present experiment, most of the OEC family protein or subunit genes were repressed at the mRNA level (Table 2). OTA treatment specifically induces decreases of OEC family protein; in this sense, damage to OEC activity in OTA-treated plants would intensify photosynthesis disorders.

**Transporters and annexin**

The regulated transport of molecules across the plasma and vacuolar membranes is a well-characterized response to abiotic stress (Jiang and Deyholos, 2006). Abundant transporters for water, sugars, cations, and other molecules were detected by DGE in response to OTA (Table 2). The OTA-responsive aquaporins, cation/H+ exchanger, and vesicle-mediated transport proteins were also up-regulated transcripts in the present data. Among the remaining antiporters, as well as sugar transporters, ABC, and multi-antimicrobial extrusion (MATE)-like efflux carriers, specific transcripts were induced by OTA treatment (Table 2). Within the MATE family in particular, seven detectable genes were induced >2-fold by OTA, whereas no single MATE gene was equivalently repressed. The large proportion of induced genes within the MATE family, plus the magnitude of their induction, suggests an important role for MATE efflux carriers in the Arabidopsis response to OTA. Almost all of the detectable syntaxins of plants were induced by OTA stress in the present data. The Arabidopsis syntaxin SYP121 resides in the plasma membrane and forms heterooligomeric complexes for vesicle-mediated secretory defence together with the adaptor SNAP33 and endomembrane-anchored VAMP722. The plasma membrane-resident PEN3 ABC transporter acts in a second pathway and has been implicated
in cytoplasmic synthesis, transporting unknown small molecules across the plasma membrane (Bednarek et al., 2009). These results reveal that OTA activates a relatively large transport function of the plasma membrane; in other words, OTA stress signalling is amplified by transmembrane signal transduction.

The annexin AnnAt1 (1.8) was up-regulated in the proteomic analysis, and its mRNA levels displayed a similar expression trend. The gene encoding AnnAt1 (AT1G35720) was expressed 1.29-fold under OTA stress (Fig. 7, Table 1). Annexins act as targets of calcium signals in eukaryotic cells, and recent results suggest that they play an important role in plant stress responses. AnnAt1 mRNA levels were up-regulated in Arabidopsis leaves by most of the stress treatments applied (Konopka-Postupolska et al., 2009). Annexins from Arabidopsis have peroxidase-like activity, and the expression of some annexins is induced by factors affecting the redox state of the cell. In addition, annexins may contribute to the regulation of ROS levels during the oxidative burst (Gidrol et al., 1996; Gorecka et al., 2005). These experimental data are sufficient to demonstrate that the biological membrane system is a pivotal organelle in response to OTA exposure that senses stress and conducts transmembrane signals. Furthermore, MATE efflux carriers and syntaxin residing in the plasma membrane can transport unknown small molecules across the plasma membrane under OTA stress.

In conclusion, this is the most comprehensive report to date of transcriptomic and proteomic analyses in response to OTA treatment in Arabidopsis leaves, and the results showed that OTA induced ROS generation and activated xenobiotic detoxification, plant hormones participated in response to OTA exposure, increased ROS caused antioxidant enzyme defence responses and compromised photosynthesis and photorespiration, and ROS resulted in dynamic changes in transcription factors, nuclear damage, and frequent biological membrane transport as signalling molecules. A hypothetical model of the regulation network activated in response to OTA in Arabidopsis leaf cells is shown in Fig. 8. The present study contributes to the understanding of the signalling transduction mechanism that modulates OTA phytotoxicity, facilitates mapping of regulatory networks, and extends the ability to improve OTA tolerance in Arabidopsis.

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**Fig. 7.** Real-time PCR analyses of the genes CRN1, CAB, SHM1, PGK, PsbP-1, AnnAt1, and the internal reference gene Actin2. Gene expression ratios (relative to the control treatment) are shown. Standard errors of the mean are shown (n=3).

**Fig. 8.** Hypothetical model of the regulatory network in response to OTA in Arabidopsis leaf cells.
Supplementary data

Supplementary data are available at JXB online.

Table S1. Gene-specific primers used to verify the DGE data in real-time PCR analyses. Results of the microarray analysis using ROBIN v. 1.1.5 with standard settings.

Table S2. Protein ratios in response to OTA exposure identified using 2DE and the Independent Samples Test of the SPSS software.

Table S3. Gene Ontology (GO) term enrichment analyses of the digital gene expression profiles.

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