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Effect of herbicidal application of 2,4-dichlorophenoxyacetic acid in *Arabidopsis*

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Abstract The whole genome expression pattern of *Arabidopsis* in response to the auxinic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was evaluated using the Affymetrix ATH1-121501 array. *Arabidopsis* plants were grown in vitro and were exposed to 1 mM 2,4-D for 1 h, after which time gene transcription levels were measured. In response to the treatment 148 genes showed increased levels of transcription and concurrently 85 genes showed decreased levels of transcript. Genes which showed significant change in transcription levels belonged to the following functional categories: transcription, metabolism, cellular communication and signal transduction, subcellular localisation, transport facilitation, protein fate, protein with binding function or cofactor requirement and regulation of/interaction with cellular environment. Interestingly 25.3% of the genes regulated by the treatment could not be classified into a known functional category. The data obtained from these experiments were used to assess the current model of auxinic herbicide action and indicated that 2,4-D not only modulates the expression of auxin, ethylene and abscisic acid (ABA) pathways but also regulates a wide variety of other cellular functions.

Keywords 2,4-Dichlorophenoxyacetic acid · Auxinic herbicide · *Arabidopsis* · Microarray-ATH1-121501

Experiment station: Plant Biotechnology Centre, Primary Industries Research Victoria, Department of Primary Industries, La Trobe University, Bundoora, Vic. 3086, and the Victorian Microarray Technology Consortium VMTC.

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Introduction

Auxinic herbicides have been widely used over the past few decades to control dicot weeds in cereal crops and are grouped into different classes based on their chemical nature: phenoxycarboxylic acids [e.g. 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA)], benzoic acids (e.g. dicamba), pyridines (e.g. picloram) and quinoline carboxylic acids (e.g. quinclorac and quinmerac). Auxinic herbicides, as their name suggests, mimic auxins. At low concentrations they induce cell division and elongation while at higher concentrations they have inhibitory effects on growth and development (Sterling and Hall 1997; Grossmann 2000; Zheng and Hall 2001). Some of the abnormalities observed with increasing concentrations of auxins and auxin-like herbicides include epinasty, leaf abscission, and abnormal elongation of root and aerial structures leading to senescence (Grossmann 2000). These features of auxin overdose have led to the adoption of synthetic auxins as herbicides in agriculture (Cobb 1992; Sterling and Hall 1997; Grossmann 1998).

Although the phenotypic response to auxin-like herbicides is well characterised, the biochemical and molecular basis of their action is less well understood. Recent studies have proposed that ethylene is induced in response to auxin-like herbicides (Grossmann 2000; Zheng and Hall 2001), and that ethylene in turn triggers abscisic acid (ABA) biosynthesis (Grossmann and Hansen 2001). This induction of ethylene is due to increased expression of the gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which catalyses the rate limiting step in

ethylene biosynthesis (Yang and Hoffman 1984; Woeste et al. 1999). Studies in cleavers (*Galium aparine* L.) support the proposal that ABA biosynthesis is triggered in response to auxin-induced ethylene biosynthesis (Hansen and Grossmann 2000), and this increase in ABA biosynthesis is mediated by 9-*cis*-epoxycarotenoid dioxygenase, a key regulator of the ABA biosynthesis pathway (Cutler and Krochko 1999; Qin and Zeevaart 1999; Hansen and Grossmann 2000). Further cell damage and death is thought to be due to the synthesis of cyanide, formed as a co-product of ethylene biosynthesis during oxidation of ACC by ACC oxidase (Grossmann 1996).

In summary, a scheme for the mode of action of auxinic herbicides was proposed from research with auxin-sensitive dicot cleavers; a cascade of reactions, initiated by an increase in ethylene levels, mediated via de novo synthesis of ACC synthase results in an increase of ABA levels. This increase in ABA is rapid, occurring within 5 h of root treatment, and that ABA accumulation results in growth inhibition and other morphological abnormalities (Hansen and Grossmann 2000). This phenomenon is sometimes referred to as “auxin overdose” (Grossmann 2000). Detailed studies of kinetics of the auxin response in shoots of treated *G. aparine* plants indicated increased levels of ACC synthase activity, with corresponding increases in ACC and ethylene 2 h after root treatment with 0.5 mM indole acetic acid (IAA; Hansen and Grossmann 2000) clearly indicating rapid signal transduction and genetic response in the plant after application of IAA to the roots.

In this study we report the results of a holistic approach to understand the effects of auxinic herbicides on plant gene expression. We have chosen to use the ATH1-121501 Affymetrix array to monitor global changes in gene expression. The data evaluate the expression of genes associated with the current model of auxinic herbicides as proposed by Hansen and Grossmann (2000). The data will be used to understand how these genome-wide changes may indicate the onset of senescence and plant death in response to 2,4-D application.

Materials and methods

Seed germination and experimental treatments

Arabidopsis thaliana ecotype Columbia plants were raised from surface-sterilised seeds in vitro on 30 ml half strength Murashige and Skoog medium supplemented with 1.5% (w/v) sucrose and vitamin and solidified with 1% phytagel (w/v) in deep petri dishes. Twelve plants per plate were maintained. Plates were kept in the dark at 4°C for 48 h and were grown in culture rooms at 22°C under a 16 h photoperiod. Plants were raised up to 14 days from day of sowing. At this stage the plants developed four rosette leaves measuring greater than 1 mm in diameter, which represents the principal growth stage 1.04 (Boyes et al. 2001). Plants at this stage were treated with 1 mM 2,4-D (pH 7.0) for a period of 1 h. Control plants were flooded with 1 ml distilled water.

Target preparation and hybridisation for microarrays

Total RNA was extracted from 100 mg plant material using the RNeasy plant mini kit (Qiagen cat. no. 74904). RNA (10 μg) was reverse transcribed to generate the first strand cDNA using Superscript II RT (Invitrogen cat. no. 18090-019) and HPLC purified T7-(dT)24 primer (Genset). Second strand synthesis was continued to generate double-stranded cDNA (ds cDNA). The ds cDNA was purified using the Phase Lock Gel (Eppendorf-5 Prime, cat. no. pl-188233). The purified ds cDNA was in vitro transcribed into cRNA using the BioArray High Yield RNA Transcript Labelling Kit (Affymetrix, Millenium Science, Australia, cat. no. 900182). Biotin label was incorporated during in vitro transcription. Labelled cRNA (20 μg) was cleaned using the RNeasy Plant Mini Kit (Qiagen cat. no. 74904) and fragmented. A hybridisation cocktail was prepared with the fragmented cRNA (target) and was spiked with alignment control oligo B2, eukaryotic hybridisation controls and background control bovine serum albumin (BSA). The target was hybridised on to probe ATH1-121501 for 16 h. The hybridised probe was stained with R-phycoerytherin streptravidin (SAPE, Molecular Probes, cat. no. S-866) and anti-strepatravidin antibody (goat) biotinylated (Vector Laboratories, cat. no. BA-0500). The stained probe was scanned using the Gene Array Scanner. All image and data analysis was performed using the Microarray Suite version 5.0 (MAS 5.0, Affymetrix). Micro DataBase version 3.0 (MicroDB 3.0) served as the interface; data mining was continued using the Data Mining Tool version 3 (DMT 3.0, Affymetrix). For biological duplication two sets of plants were separately treated and RNA extracted from each were separately used to generate cRNA. Results from both the hybridisations were used to verify the reliability of expression results.

Results and discussion

In the experiments discussed below, *Arabidopsis* plants raised in culture on half strength Murashige and Skoog (MS) medium for 14 days were exposed to 2,4-D by root irrigation to a final concentration of 1 mM. After exposure for 1 h RNA was extracted from treated seedlings and was used to assess changes in gene expression levels. The probe used was the Affymetrix *Arabidopsis* array ATH1-121501, which enabled a quantitative approach to the examination of changes in all transcripts within the *Arabidopsis* genome.

Biological duplication

Biological duplication of the treatment (1 mM 2,4-D; 1 h) was used to verify the consistency in expression pattern. The Affymetrix Microarray Suite version 5.0 (MAS 5.0) software was used for analysis. Comparison of array results indicated that 99.2% of the genes that were called present in both duplicates (13,480) showed a variation coefficient of less than 50% (Fig. 1) indicating an extremely high degree of reproducibility between the biological replicates, and scatter plot analysis of the signal values from treatment duplicates further support this, with 80% of probes on both arrays within a twofold difference (Fig. 2). This analysis supports the premise that there was low biological variability between the duplicates.

The selection of reliable signals was carried out in a systematic manner following the procedure outlined by Wang et al. (2003). Signal values were generated based on one-step Tukey's biweight estimate and detection calls were made based on the Wilcoxon's signed rank test (Affymetrix 2002). To detect those genes that increased significantly, genes that gave a detection call "P" (present) in both duplicated arrays were chosen. From the P set of genes, those that gave a change call "I" (increase) and a twofold change in both the arrays with average signal values greater than 100 in case of the treatment were considered to have significantly increased (Table 1). To detect genes that decreased in expression those that gave a P (present) call in the control were selected, and those that decreased in both duplicated arrays by a twofold difference and with signal value greater than 100 in the control were considered to have decreased significantly (Table 1). Figure 3 represents a scatter plot of signal values of the control versus average signal of the duplicated arrays of treatment.

Gene expression and functional classification

From a total of 22,810 genes on the ATH1-121501 array, 13,101 (57%) were called P in the control and 13,480 (59%) were called P in both treatments arrays. These percentages and similarity indicated reliable signals. A significant increase or decrease in signal in response to the treatment was indicated by an I (increase) or D (decrease), respectively. Expression results identified that about 1.0% (233) of all of the genes represented on the array were regulated with a greater than twofold change and signal values greater than 100. Data mining identified 148 genes up-regulated (Table 2) and 85 genes down-regulated (Table 3) in response to treatment of the plant roots with 2,4-D (1 mM for 1 h).

Functions were assigned based on automatically derived functional categories maintained by the Munich Information Center for Protein Sequences (MIPS): http://mips/.gsf.de/proj/thal/tables/tables_func_frame.html. Some of the significant categories of the up-regulated genes included cell rescue, defence and virulence, metabolism, transcription, cellular communication/signal transduction, transport facilitation and subcellular localisation (Table 2). Down-regulated genes were grouped into the following major categories: transcription, metabolism, cellular communication/signal transduction, cell fate and cell rescue, defence and virulence (Table 3). Functional classification indicated that 25.3% of the regulated genes did not belong to any known functional category (Fig. 4).

Analysis of auxin, ethylene and abscisic acid associated genes

Auxins are known to induce a series of responses related to growth and development in plants and genes have been shown to be up-regulated within 5–60 min of exposure to auxins (Abel and Theologis 1996). These early auxin response genes fall into three major categories: auxin/indole acetic acid (*Aux*/*IAA*), *SAUR* (small up-regulated proteins) and *GH3* (Hagen and Guilfoyle 1985). Members of the *Aux*/*IAA* multigene family are up-regulated by auxins (Reed 2001). Our results indicated that a number of these auxin-related genes were regulated in response to 2,4-D, confirming the auxin-like nature of 2,4-D when applied to the roots of the plants at 1.0 mM concentration. Genes encoding putative auxin-responsive proteins

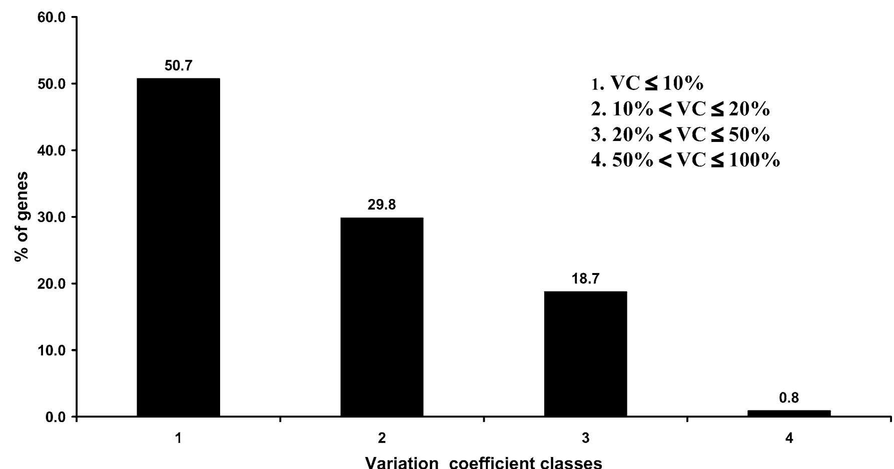

Fig. 1 Variation coefficients of 13,480 genes called present in both hybridisations of 1 mM 2,4-Dichlorophenoxyacetic acid over 1 h

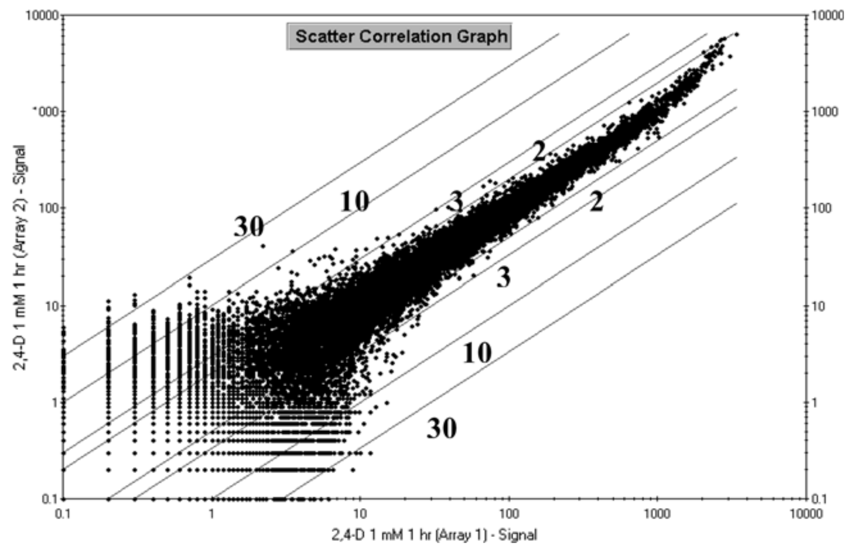

Fig. 2 Scatter plot of signal values from biological replicates of 1.0 mM 2,4-D represents reproducibility of transcript abundance measurements. Two RNA samples were separately prepared and targets generated from each of the RNA samples were individually hybridised to two arrays. Signal values from array 1 are represented on the *x*-axis and signal values from array 2 are represented on *y*-axis. The *diagonal lines* represent fold changes, which are indicated by *number* across the lines

(*IAA1*), auxin-induced proteins (*IAA5*, *IAA19*), and an auxin-regulated protein (*IAA13*) were up-regulated in response to 2,4-D. However, gene expression of a putative auxin-induced basic helix-loop-helix transcription factor and an auxin-induced protein 10A were down-regulated in this study.

In response to auxinic herbicides and high levels of auxins, ethylene biosynthesis is induced, which in turn triggers the biosynthesis of ABA. ABA subsequently plays a role in adaptive stress responses, but may also trigger growth inhibition and herbicide-like symptoms. It has been observed that in *G. aparine*, in response to IAA, the activity of ACC synthase and the levels of ACC and ethylene show a rapid but transient increase, with ACC synthase activity showing a maximal activity 3 h post-IAA application with ACC and ethylene levels peaking 5 h post-application (Hansen and Grossmann 2000).

Hansen and Grossmann (2000) observed that the activity of ACC synthase, levels of ACC and ethylene increased but had declined 5 h after treatment with 0.5 mM IAA. Hansen and Grossmann (2000) also observed an increase in ABA levels after 5 h of exposure to 0.5 mM IAA. However, in this study within 1 h of exposure to 1 mM 2,4-D, *NCED3* encoding 9-*cis*-epoxycarotenoid dioxygenase, a key regulator of ABA biosynthesis, was up-regulated but there was no change in expression of genes encoding ACC synthase or ACC oxidase.

Although 2,4-D did not appear to regulate genes involved in ethylene biosynthesis, the results did indicate regulation of genes further along the ethylene signalling cascade. The plant hormone ethylene plays a central role in plant growth, development and general stress response in *Arabidopsis*. Ethylene in turn is perceived by a group of histidine kinase receptors (*ETR1*, *ETR2*, *EIN4*, *ERS1* and *ERS2*) and downstream to these receptors is a protein kinase constitutive triple response 1 (*CTR1*). Ethylene receptors and *CTR1* are negative regulators of ethylene signalling: in the absence of ethylene the receptors are in an active state, *CTR1* binds to the receptor and inhibits the ethylene responses (Gao et al. 2003; Huang et al. 2003). Our results indicted that 2,4-D significantly down-regulated both *ERS* and *CTR1* to the same degree (signal log ratio of −1.20). This down-regulation of *ERS* and *CTR1* indicates that 2,4-D may have triggered an ethylene response, even in the absence of increased expression of genes in the ethylene biosynthesis pathway.

Downstream of *CTR1* in the ethylene response cascade there are two groups of transcription factors—ethylene insensitive/ethylene insensitive-like (*EIN3*/*EIL*) and the ethylene receptor factor (*ERF*)—which are activated by ethylene binding. *DREB2A*/*ERF4* and *AtERF8*, members of the AP2 EREBP family of transcription factors, were up-regulated in response to 2,4-D (1.0 mM; 1 h). *ERFs* interact with the *cis*-acting element GCC, found in

Table 1 Selection of genes with reliable expression

Conditions for selection	Increase	Conditions for selection	Decrease
Total no. of genes on the array	22,810	Total no. of genes on the array	22,810
No. of genes called present in both	13,480	No. of genes with a present call under control conditions	13,101
Total no. of genes with an increase call in both	647	Total no. of genes with an decrease call in both	478
Total no. of genes with an increase greater than or equal to twofold change in both	328	Total no. of genes with a decrease greater than or equal to twofold change in both	246
Total no. of genes with an increase greater than or equal to twofold change in both and signal value greater that or equal to 100	148	Total no. of genes with a decrease greater than or equal to twofold change in both and signal value greater that or equal to 100 in control	85

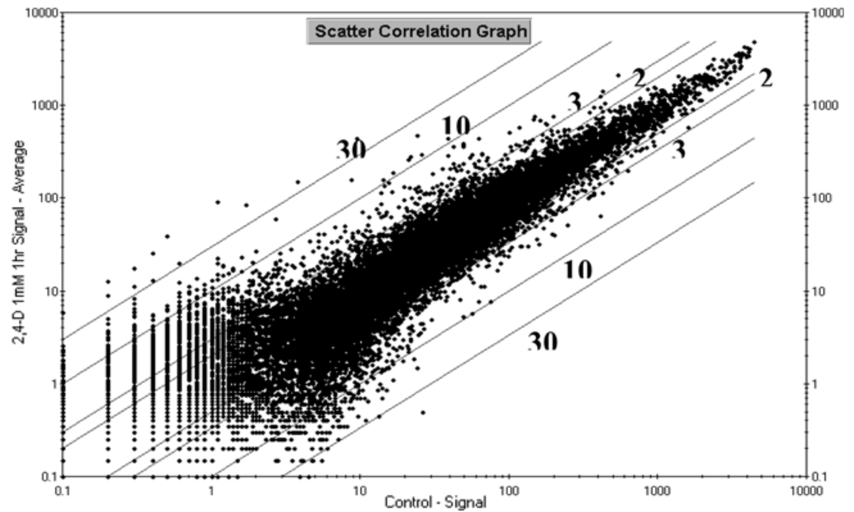

Fig. 3 Scatter plot of control signal values versus average signal of treatment (1 mM 2,4-D; 1 h). Control values on *x*-axis and treatment on *y*-axis. Fold change is represented in *bold numbers* (same as in Fig. 2)

defence-related genes, and regulate the stress response (Fujimoto et al. 2000). Regulation of *ERF* in response to biotic and abiotic stress may occur in an ethylene-independent manner and the increase in expression of *AtERF4* and *AtERF8* in response to 2,4-D might have been due to the onset of stress. Members of the ERF family are known to be differentially regulated in response to ethylene and other abiotic stresses (Fujimoto et al. 2000). *AtERF4* is a transcriptional repressor of stress response genes. Both *AtERF4* and *AtERF8* function as repressors of stress signalling. The up-regulation of *AtERF4* and *AtERF8* is possibly a result of stress induced by 2,4-D application (Fujimoto et al. 2000).

Hansen and Grossmann (2000) also reported that 5 h after IAA application both xanthoxal and ABA levels show marked elevation, with levels remaining high until 25 h post-incubation (Hansen and Grossmann 2000). The biosynthesis of ABA involves 9-*cis*-epoxycarotenoid dioxygenase, the enzyme which cleaves 9-*cis*-xanthophyll and 9-*cis*-neoxanthin precursors for ABA aldehyde and ABA. Results in this study indicated that *NCED3*, the gene encoding 9-*cis*-epoxycarotenoid dioxygenase, was strongly up-regulated in response to root application of 1 mM 2,4-D. This response was rapid, mRNA levels had increased by 1 h post-application, and the signal log ratio for *NCED3* was one of the highest observed in the entire data set, indicating significant transcription relative to the control levels. The up-regulation of *NCED3* within 1 h of application of 2,4-D may be due to the high concentration of 2,4-D applied, as Hansen and Grossmann (2000) observed an increase in ABA levels only after 5 h of exposure to 0.5 mM IAA. The increased level of

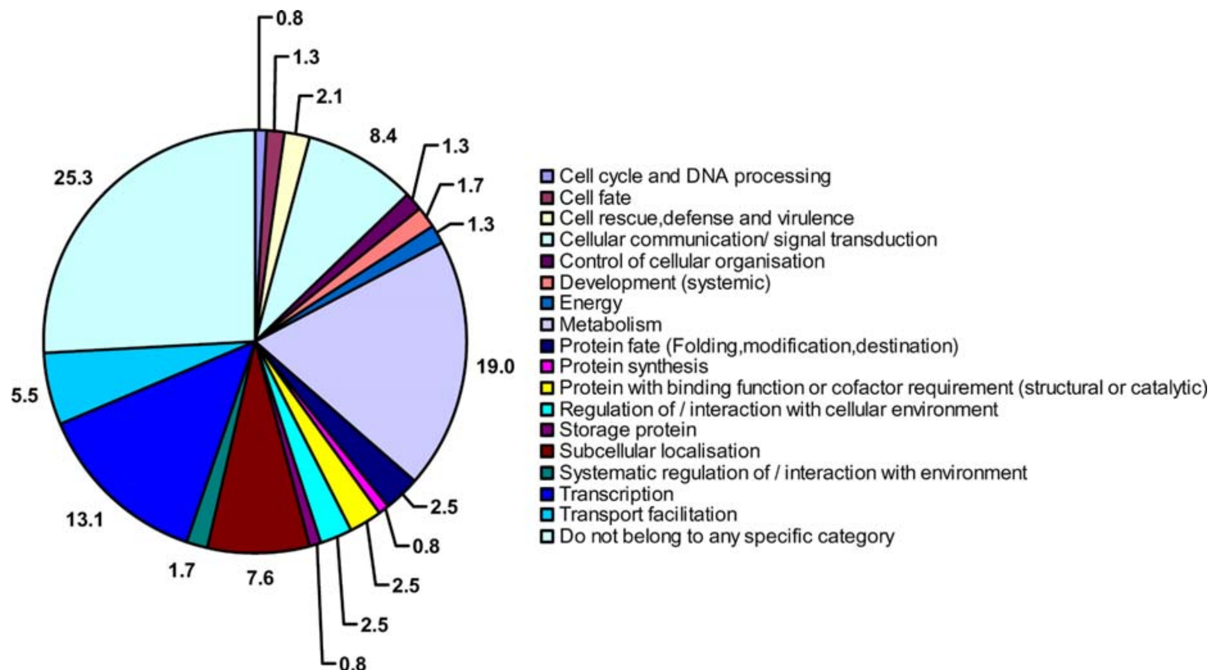

Fig. 4 Percentage representation of functional categories of genes regulated by 2,4-D 1 mM over 1 h

Table 2 A representation of genes that were up-regulated

Probe set ID	AGI ID	Average signal log ratio	Brief sequence description
Cell fate			
257280_at	At3g14440	4.47	9-*cis*-epoxycarotenoid dioxygenase (NCED3/NC1)
Cell rescue, defence and virulence			
252102_at	At3g50970	1.27	Dehydrin Xero2
249645_at	At5g36910	1.48	Thionin Thi2.2
Cellular communication or signal transduction			
267550_at	At2g32800	2.06	Putative protein kinase
267083_at	At2g41100	1.52	Calmodulin-like protein
266749_at	At2g47060	1.18	Putative protein kinase
263478_at	At2g31880	1.02	Putative receptor-like protein kinase
259922_at	At1g72770	1.64	Protein phosphatase 2C (AtP2C-HA)
259231_at	At3g11410	2.47	Protein phosphatase 2C (PP2C)
255502_at	At4g02410	2.12	Contains similarity to a protein kinase domain
254256_at	At4g23180	1.96	Serine/threonine kinase-like protein
253994_at	At4g26080	2.37	Protein phosphatase ABI1
247723_at	At5g59220	3.39	Protein phosphatase 2C-like ABA-induced protein phosphatase 2C
245765_at	At1g33600	1.23	Hypothetical protein similar to putative disease resistance protein
Development (systemic)			
261564_at	At1g01720	3.20	NAC domain protein
259705_at	At1g77450	3.90	GRAB1-like protein
247706_at	At5g59480	1.87	Putative ripening-related protein
247351_at	At5g63790	2.40	Putative protein contains similarity to NAC-domain
Energy			
265742_at	At2g01290	1.24	Putative ribose 5-phosphate isomerase
259875_s_at	At1g76690	2.10	12-oxophytodienoate reductase (OPR2)
245734_at	At1g73480	2.61	Lysophospholipase homolog
Metabolism			
265075_at	At1g55450	1.61	Hypothetical protein similar to embryo-abundant protein
264931_at	At1g60590	1.12	Polygalacturonase
264904_s_at	At2g17420	1.13	Putative thioredoxin reductase
260955_at	At1g06000	1.15	Unknown protein contains similarity to UDPG glucosyltransferase
260727_at	At1g48100	3.47	Polygalacturonase PG1(Mahalingam et al. 1999)
260602_at	At1g55920	1.42	Serine acetyltransferase (Ruffet et al. 1995)
260393_at	At1g73920	1.45	Putative lipase
258507_at	At3g06500	1.79	Neutral invertase
258114_at	At3g14660	1.20	Putative cytochrome P450
256994_s_at	At3g25830	1.07	Limonene cyclase
256861_at	At3g23920	1.67	Beta-amylase
256589_at	At3g28740	2.27	Cytochrome P450
255622_at	At4g01070	1.01	Putative flavonol glucosyltransferase
254759_at	At4g13180	1.96	Short-chain alcohol dehydrogenase
254343_at	At4g21990	1.74	PRH26 protein
253638_at	At4g30470	1.80	Cinnamoyl-CoA reductase
253268_s_at	At4g34135	3.12	Glucosyltransferase-like protein immediate-early salicylate-induced glucosyltransferase
253073_at	At4g37410	1.53	Cytochrome P450 monooxygenase-like protein cytochrome P450 monooxygenase CYP91A2
252943_at	At4g39330	1.22	Cinnamyl-alcohol dehydrogenase CAD1
252363_at	At3g48460	1.34	Lipase-like protein lipase Arab-1
251775_s_at	At3g55610	1.64	Delta-1-pyrroline-5-carboxylate synthetase (P5CS1)

Table 2 (continued)

Probe set ID	AGI ID	Average signal log ratio	Brief sequence description
251658_at	At3g57020	1.17	Putative protein strictosidine synthase
250549_at	At5g07860	1.45	*N*-hydroxycinnamoyl benzoyltransferase
249599_at	At5g37990	1.92	Putative protein AtPP protein
249411_at	At5g40390	2.12	Raffinose synthase
249325_at	At5g40850	1.32	Urophorphyrin III methylase
246468_at	At5g17050	1.28	UDP glucose:flavonoid 3-*o*-glucosyltransferase
246463_at	At5g16970	1.08	Quinone oxidoreductase
246126_at	At5g20070	1.20	Putative protein predicted protein, *Oryza sativa*
245277_at	At4g15550	1.36	Glucosyltransferase like protein
Protein fate (folding, modification, destination)			
254204_at	At4g24160	1.50	Putative protein CGI-58 protein
Protein synthesis			
258735_at	At3g05880	1.24	Low temperature and salt responsive protein (LT16A)
Protein with binding function or cofactor requirement (structural or catalytic)			
256245_at	At3g12580	2.20	Heat shock protein 70 (hsp70)
254784_at	At4g12720	1.94	Growth factor like protein antisense basic fibroblast growth factor
249798_at	At5g23730	1.43	Putative protein contains similarity to photomorphogenesis repressor protein
Regulation of/interaction with cellular environment			
263831_at	At2g40300	1.28	Putative ferritin
251735_at	At3g56090	1.50	Putative protein ferritin 2 precursor
251109_at	At5g01600	1.66	Ferritin 1 precursor
Subcellular localisation			
266296_at	At2g29420	2.59	Putative glutathione S-transferase
264436_at	At1g10370	1.30	Putative glutathione S-transferase TSI-1
258452_at	At3g22370	1.86	Alternative oxidase 1a precursor
258086_at	At3g25860	1.06	Dihydrolipoamide S-acetyltransferase
256453_at	At1g75270	1.41	GSH-dependent dehydroascorbate reductase 1
255543_at	At4g01870	2.83	Predicted protein of unknown function similar to bacterial tolB proteins
254920_at	At4g11220	1.13	Putative protein; 24 kDa seed maturation protein
254085_at	At4g24960	2.63	Abscisic acid-induced-like protein; abscisic acid-induced protein HVA22
249267_at	At5g41600	1.53	Putative protein contains similarity to 24 kDa seed maturation protein
248607_at	At5g49480	2.09	NaCl-inducible Ca^{2+}-binding protein-like; calmodulin-like
Systematic regulation of/interaction with environment			
263786_at	At2g46370	1.36	Putative auxin-responsive protein
Transcription			
267028_at	At2g38470	1.51	Putative WRKY-type DNA binding protein
263379_at	At2g40140	1.37	Putative CCCH-type zinc finger protein; also an ankyrin-repeat protein
262590_at	At1g15100	1.25	Putative RING-H2 zinc finger protein
261766_at	At1g15580	4.26	Auxin-induced protein IAA5
261315_at	At1g53170	2.21	AP2 domain-containing protein, putative similar to AP2 domain-containing protein RAP2.5 (AtERF8)
259626_at	At1g42990	1.29	bZIP transcription factor
258399_at	At3g15540	2.14	Early auxin-induced protein, IAA19
257262_at	At3g21890	3.18	Zinc finger protein
257053_at	At3g15210	1.61	Ethylene responsive element binding factor 4 (AtERF4)
255788_at	At2g33310	1.32	Auxin regulated protein (IAA13)
253263_at	At4g34000	1.39	Abscisic acid responsive elements-binding factor(ABF3)

Table 2 (continued)

Probe set ID	AGI ID	Average signal log ratio	Brief sequence description
253038_at	At4g37790	1.66	Homeobox protein HAT22
251745_at	At3g55980	1.50	Putative protein zinc finger transcription factor (PEI1)
251272_at	At3g61890	2.44	Homeobox-leucine zipper protein ATHB-12
250304_at	At5g12110	1.48	Elongation factor 1B alpha-subunit
248801_at	At5g47370	2.07	Homeobox-leucine zipper protein-like
248764_at	At5g47640	1.90	Putative protein contains similarity to CCAAT-box-binding transcription factor
247452_at	At5g62430	1.75	H-protein promoter binding factor-like protein
245397_at	At4g14560	1.42	Auxin-responsive protein IAA1
Transport facilitation			
266718_at	At2g46800	1.25	Putative zinc transporter
265444_s_at	At2g37180	1.58	Aquaporin (plasma membrane intrinsic protein 2C) water channel protein in plasma membrane
264000_at	At2g22500	1.93	Putative mitochondrial dicarboxylate carrier protein
262935_at	At1g79410	2.00	Hypothetical protein similar to putative transporter
262883_at	At1g64780	1.42	Ammonium transporter
261650_at	At1g27770	1.69	Envelope Ca^{2+}-ATPase identical to envelope Ca^{2+}-ATPase
258181_at	At3g21670	1.28	Nitrate transporter
252671_at	At3g44190	1.58	Putative protein dehydrogenases
251785_at	At3g55130	1.17	ABC transporter-like protein breast cancer resistance protein 1 BCRP1
247593_at	At5g60790	1.52	ABC transporter homolog PnATH-like PnATHABC transporter homolog
Do not belong to any specific category			
255795_at	At2g33380	3.03	Putative calcium-binding EF-hand protein
251084_at	At5g01520	1.77	Putative protein putative proteins—*Arabidopsis thaliana*
264758_at	At1g61340	2.76	Late embryogenesis abundant protein
263098_at	At2g16005	2.64	Expressed protein
259773_at	At1g29500	1.31	Auxin-induced protein
256603_at	At3g28270	1.05	Unknown protein similar to At14a protein
254850_at	At4g12000	2.61	Putative protein predicted proteins—*Arabidopsis thaliana*
254050_s_at	At4g25670	1.95	Hypothetical protein
251039_at	At5g02020	2.02	Putative protein hypothetical protein T6H20.90
250438_at	At5g10580	1.25	Putative protein predicted protein
248337_at	At5g52310	3.47	Low-temperature-induced protein 78
245885_at	At5g09440	1.26	Putative protein phi-1—*Nicotiana tabacum*
262098_at	At1g56170	1.12	Transcription factor
259426_at	At1g01470	1.43	Hypothetical protein contains similarity to 1-phosphatidylinositol-4-phosphate 5-kinase(AtPIP5K1)
249078_at	At5g44070	1.56	Phytochelatin synthase
247488_at	At5g61820	1.94	Putative protein MtN19
244996_at	Not Known	1.39	Ribosomal protein S2
244975_at	Not known	1.38	PSII 10 kDa phosphoprotein
244962_at	Not known	1.36	NADH dehydrogenase ND4

transcription of this gene implies that ABA may accumulate beyond normal physiological levels, which may in turn cause a variety of physiological responses leading to cell death and tissue damage, consistent with the auxin-overdose hypothesis proposed by Grossmann (2000). The gene encoding 9-*cis*-epoxycarotenoid is also known to be regulated in response to other forms of physiological stress (Cutler and Krochko 1999; Qin and Zeevaart 1999) indicating that induction of this gene may be part of an overall stress response by the plant as a result of the high levels of 2,4-D application used in the present study.

Hansen and Grossmann (2000) also confirmed that there was no increase in activity of levels of violaxanthin, neoxanthin and β-carotene (xanthophyll cycle) in response to high levels of auxin application. Our results supported

Table 3 A representation of genes that were down-regulated

Probe set ID	AGI ID	Average signal log ratio	Sequence description
Cell cycle and DNA processing			
257701_at	At3g12710	−1.24	Unknown protein contains similarity to 3-methyladenine-DNA glycosidase I
249008_at	At5g44680	−1.38	Putative protein contains similarity to DNA-3-methyladenine glycosylase I
Cell fate			
253161_at	At4g35770	−1.47	Senescence-associated protein sen1
Cell rescue, defence and virulence			
253125_at	At4g36040	−1.12	DnaJ-like protein
Cellular communication/signal transduction			
261308_at	At1g48480	−1.27	Protein kinase
256516_at	At1g66150	−1.01	Receptor protein kinase (TMK1)
254770_at	At4g13340	−1.38	Extensin-like protein
252992_at	At4g38520	−1.25	Putative protein phosphatase-2c
251017_at	At5g02760	−1.48	Protein phosphatase-like protein protein phosphatase 2C homolog
250911_at	At5g03730	−1.20	Serine/threonine-protein kinase CTR1
248910_at	At5g45820	−1.68	Serine threonine protein kinase
Control of cellular organisation			
255822_at	At2g40610	−1.23	Putative expansin
254818_at	At4g12470	−1.96	pEARLI 1-like protein
253061_at	At4g37610	−1.26	Putative protein SPOP
Metabolism			
261825_at	At1g11545	−1.33	Endo-xyloglucan transferase
258552_at	At3g07010	−1.32	Putative pectate lyase
257315_at	At3g30775	−1.70	Proline oxidase, mitochondrial precursor (osmotic stress-induced proline dehydrogenase; Kiyosue et al. 1996)
257203_at	At3g23730	−1.87	Xyloglucan endotransglycosylase
255433_at	At4g03210	−1.42	Putative xyloglucan endotransglycosylase
254363_at	At4g22010	−1.10	Pectinesterase
253666_at	At4g30270	−1.04	Xyloglucan endo-1,4-beta-D-glucanase precursor
249037_at	At5g44130	−1.43	Putative protein contains similarity to surface protein
248622_at	At5g49360	−1.34	Xylosidase
247925_at	At5g57560	−1.56	TCH4 protein
247266_at	At5g64570	−1.75	Beta-xylosidase
246114_at	At5g20250	−1.07	Seed imbitition protein-like seed imbitition protein Sip1
245777_at	At1g73540	−2.26	Unknown protein contains similarity to diphosphoinositol polyphosphate phosphohydrolase
Protein fate (folding, modification, destination)			
266106_at	At2g45170	−1.03	Putative microtubule-associated protein
254688_at	At4g13830	−1.25	DnaJ-like protein
251899_at	At3g54400	−1.01	Nucleoid DNA-binding
245098_at	At2g40940	−1.20	Ethylene response sensor (ERS)
Protein synthesis			
245886_at	At5g09510	−1.05	Ribosomal protein S15
Protein with binding function or cofactor requirement (structural or catalytic)			
259950_at	At1g71410	−1.34	Hypothetical protein contains protein kinase domain
Regulation of/interaction with cellular environment			
264751_at	At1g23020	−1.46	Putative superoxide-generating NADPH oxidase flavocytochrome highly similar FRO1 and FRO2
247288_at	At5g64330	−1.25	Non-phototropic hypocotyl 3
Storage protein			
249073_at	At5g44020	−1.25	Vegetative storage protein-like

Table 3 (continued)

Probe set ID	AGI ID	Average signal log ratio	Sequence description
245925_at	At5g28770	−1.13	bZIP transcription factor family protein similar to seed storage protein opaque-2(bZIP family)
Subcellular localisation			
264097_s_at	At1g79010	−1.10	NADH dehydrogenase
260957_at	At1g06080	−1.21	Delta 9 desaturase
250752_at	At5g05690	−1.17	Cytochrome P450 90A1
245637_at	At1g25230	−1.15	Hypothetical protein similar to putative purple acid phosphatase precursor
Transcription			
263064_at	At2g18160	−1.41	Putative bZIP transcription factor
259751_at	At1g71030	−2.59	Putative transcription factor similar to myb-related transcription factor 24
258434_at	At3g16770	−1.15	AP2 domain containing protein RAP2.3
251705_at	At3g56400	−1.02	DNA-binding protein like DNA-binding protein 4 WRKY4
251373_at	At3g60530	−1.44	GATA transcription factor 4
251365_at	At3g61310	−1.12	Putative DNA-binding protein
246932_at	At5g25190	−1.70	Ethylene-responsive element
246011_at	At5g08330	−1.17	Putative protein auxin-induced basic helix-loop-helix transcription factor
Transport facilitation			
263777_at	At2g46450	−1.18	Putative cyclic nucleotide-regulated ion channel protein
259839_at	At1g52190	−1.35	Peptide transporter, putative similar to peptide transporter PTR2-B
Do not belong to any specific category			
255064_at	At4g08950	−2.37	Putative phi-1-like phosphate-induced protein
252965_at	At4g38860	−1.23	Putative auxin-induced protein; auxin-induced protein 10A
246917_at	At5g25280	−1.03	Serine-rich protein
267076_at	At2g41090	−1.22	Calcium binding protein (CaBP-22)
262958_at	At1g54410	−1.20	Water stress-induced protein
260037_at	At1g68840	−1.97	Putative DNA-binding protein (RAV2-like)
257832_at	At3g26740	−1.46	Light regulated protein (Reimmann and Dudler 1993)
256438_s_at	At3g11120	−1.34	Ribosomal protein L41
244967_at		−1.54	PSI J protein

this observation, as there was no significant change in expression of genes involved in the xanthophyll cycle, suggesting that it is the increase in levels of *NCED3* which leads to increased ABA formation.

A number of genes encoding ABA-induced proteins (Seki et al. 2002) indicated increased levels of gene transcription in response to 2,4-D (e.g. ABA induced protein phosphatase 2C and protein phosphatase *ABI1*, which are both involved in signal transduction). Recent studies by Hoth et al. (2002) using mutant *abi1* have identified *ABI1* as an ABA-responsive gene. Gene *ABF3*, an ABA-responsive-element (ABRE) binding factor that has been shown to play a role in ABA signalling in response to stress (Kang et al. 2002), was induced in this study. Another ABA-response-element binding factor induced as a response to 2,4-D was the homeobox-leucine *ATHB-12* transcription factor, previously induced in response to ABA (Lee et al. 2001). The *HVA22* gene, which encodes another ABA and stress-induced protein (Chen et al. 2002) and classified by MIPS as involved in subcellular localisation was shown to be up-regulated by 2,4-D, as was dehydrin Xero2 protein, classified as involved in cell rescue, defence and virulence.

Therefore, the results obtained via analysis of the entire transcriptome of *Arabidopsis* suggest that exposure to high concentrations of auxinic herbicides (1.0 mM 2,4-D) regulated genes involved in auxin response (*IAA1*, *IAA5*, *IAA13*, *IAA19*), ethylene signalling (*ERS*, *CTR1*, *AtERF4*, *AtERF8*), ABA biosynthesis (key regulatory gene *NCED3*) and ABA signalling and response (*ABF3*, *ABI1*). A schematic representation of the key changes in gene expression in *Arabidopsis* as a response to application of 1.0 mM 2,4-D to plant roots is presented in Fig. 5. Our results identified that 2,4-D down-regulated *ERS* and *CTR1*, negative regulators of ethylene response, and up-regulated *AtERF4* and *AtERF8*, repressors of GCC-motif-containing stress response genes. The up-regulation of *AtERF4* and *AtERF8* may possibly be due to stress. We also found that 2,4-D induced *NCED3*, a key regulator of ABA biosynthesis. The previously observed increase in

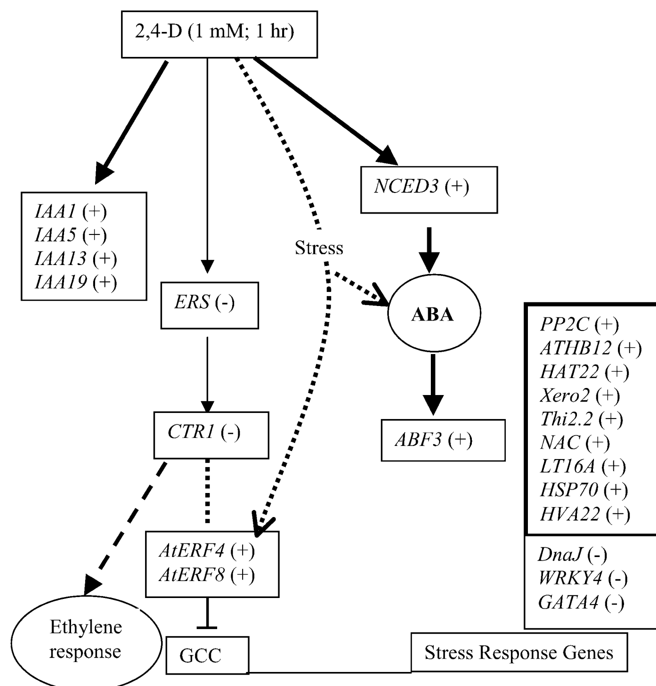

Fig. 5 A schematic representation of gene expression in response to 2,4-D (1.0 mM; 1.0 h). *Thick arrows* indicate up-regulation and *thin arrows* indicate down-regulation of genes indicated in the *boxes*. *Dotted arrow* indicate the possible path of regulation. *AtERF4* and *AtERF8* are repressors of GCC-motif-containing stress response genes. Up-regulated genes (+); down-regulated genes (−)

ABA levels may also be a response to stress. The study indicated up-regulation of *ABF3*, associated with ABA signalling and also observed the regulation of ABA response genes.

In response to stresses such as drought or salinity, it has been observed that plants tend to accumulate the osmolytes proline, glycine betaine or sugar alcohols (Yoshiba et al. 1999). Microarray analysis indicated regulation of genes encoding enzymes involved in osmolyte and osmoprotectant biosynthesis or modulation in response to root application of 2,4-D. The enzyme delta-1-pyrroline-5-carboxylate synthetase had been previously shown to play a role in ABA and salt stress-induced proline accumulation in *Arabidopsis* (Abraham et al. 2003). The results in this study, consistent with the study by Abraham et al. (2003), indicated that the gene *P5CS1* encoding delta-1-pyrroline-5-carboxylate synthetase, the rate limiting enzyme in proline biosynthesis, was up-regulated by 2,4-D. Concomitant with this was the observed down-regulation of a gene encoding proline oxidase. The gene encoding raffinose synthase involved in the synthesis of osmoprotectants, which has been previously identified to be induced during senescence (Buchanan-Wollaston et al. 2003), was up-regulated by 2,4-D. The results indicated an up-regulation of *AtPIP5K1* encoding 1-phosphatidylinositol-4-phosphate 5-kinase, also induced under water stress and in response to ABA (Mikami et al. 1998).

The expression results indicated the up-regulation of genes encoding glutathione transferases, known to be involved in detoxification (Prade et al. 1998) and which are also auxin response genes (Abel and Theologis 1996). Cytochrome P450 enzymes (Cyt P450) constitute the largest group of plant proteins that determine herbicide tolerance and selectivity (Werck-Reichhart et al. 2000), and are also involved in the biosynthesis of defence-related compounds, signalling of gibberellins and jasmonates (Maughan et al. 1997) and pigment biosynthesis. Results from this study identified the up-regulation of genes encoding cytochrome P450 and cytochrome P450 monooxygenase (*CYP91A2*) and the down-regulation of cytochrome P450 90A1 (*CYP90A1*). *CYP90A1* is involved in brassinosteroid biosynthesis (Bancos et al. 2002). Further studies on the role of these cytochrome P450s may suggest an involvement in the mode of action of 2,4-D.

Jasmonic acid (JA) and salicylic acid have well documented roles in plant defence and signalling (Liechti and Farmer 2003). Oxylipin-12-oxophyto-dienoic acid (OPDA) is involved in signal transduction and is a precursor in jasmonic acid biosynthesis, and has been reported to control a number of cellular processes (Schaller and Weiler 1997; Schaller et al. 2000). The three isozymes of 12-oxophytodienoate reductase (*OPR1*, *OPR2*, *OPR3*) are involved in the conversion of OPDA to JA. Brassinosteroids are known to up-regulate the expression at the *OPR3* gene (Mussig et al. 2002) and the genes *OPR1* and *OPR2* are up-regulated in response to wounding and UV light (Biesgen and Weiler 1999). Expression of the gene encoding 12-oxophytodienoate reductase (*OPR2*) was induced in response to 1.0 mM 2,4-D with 1 h application.

The expression of a number of rescue, defence and virulence-related genes were also regulated in response to 2,4-D. The induction of dehydrin *Xero2* gene is consistent with reports of induction of this gene by endogenous ABA (Rouse et al. 1996). The thionin gene, *Thi2.2*, associated with signalling, cell death and systemic acquired resistance (SAR) pathways (Nibbe et al. 2002) also showed induction after 2,4-D application. Also of note was the induction of ferritin genes, reported to show differential regulation in response to iron as well as a variety of stresses and senescence (Pic et al. 2002; Tarantino et al. 2003). The genes encoding a low temperature and salt responsive protein *LT16A*, low temperature-induced protein 78 and the heat shock protein *HSP70* were also up-regulated as a response to 2,4-D. A gene encoding a leucine-rich repeat family protein involved in disease resistance was also up-regulated in this study. This study is the first to report the up-regulation of resistance/defence genes in response to auxinic herbicides. The results suggest that certain genes regulated by the herbicidal application of 2,4-D are also involved in response to stress. Cheong et al. (2002) described the overlapping gene expression patterns in response to wounding, pathogen, abiotic stress and hormonal response.

2,4-D and senescence

Senescence is induced prematurely in plants as a protective mechanism for survival against various forms of stress. Senescence is one of the mechanisms that plants have evolved for survival: to enhance chances of survival the plant triggers localized cell death. Some of the well-characterized metabolic processes observed to occur during senescence include chlorophyll, protein and lipid degradation (Buchanan-Wollaston et al. 2003). In this study, we have induced plant senescence by herbicidal applications of 1.0 mM 2,4-D. Genes encoding xyloglucan endotransglycosylases (*meri5B* and *TCH4*) and others were down-regulated in response to 1.0 mM 2,4-D. Xyloglucan endotransglycosylases catalyse the cleavage of xyloglucan leading to the loosening of cell walls and as a result promote growth. Xyloglucan endotransglycosylases (*meri5B* and *TCH4*) were induced by auxins and brassinosteroids (Xu et al. 1996). The gene *meri5B*, similar to *BRU1*, was up-regulated in response to brassinosteroids (Mussig et al. 2002) to promote cell elongation and growth. The results indicated down-regulation of *meri5B* and *TCH4* at 1.0 mM 2,4-D, suggesting inhibition of cell growth.

Senescence is also accompanied by cell wall degradation and membrane disintegration (Buchanan-Wollaston et al. 2003). Expansin proteins are associated with cell elongation and wall organisation (Goda et al. 2002) and the results of this study indicated down-regulation of a gene encoding a putative expansin in response to 2,4-D. The genes encoding pectinesterases, xylosidase and beta-xylosidase are involved in cell wall metabolism (Micheli 2001; Goujon et al. 2003) and were down-regulated in this study. At the same time there was up-regulation of genes encoding polygalacturonase, a well-characterized cell wall modifying enzyme (Mahalingam et al. 1999). The results suggest that 2,4-D (1.0 mM; 1.0 h) represses cell elongation and growth.

Lipids are a major component of the cell membrane and are degraded by lipases during senescence (Buchanan-Wollaston et al. 2003). The results indicated up-regulation of a gene encoding lipase, again consistent with previous studies investigating gene expression during senescence (Buchanan-Wollaston et al. 2003). Interestingly the results indicated that a gene encoding lipid transport protein pEARL1 was down-regulated. According to the MIPS classification the pEARL proteins are involved in control of cellular organisation. The herbicide 2,4-D, at 1.0 mM, has triggered the degradation of the membranes. Although 2,4-D (1 mM; 1 h) inhibited growth and triggered cell degradation there was significant down-regulation of the gene encoding the senescence-induced protein *sen1* (Oh et al. 1996). Results also indicated the down-regulation of transcription factor *WRKY4*, which has been reported to be induced during senescence (Chen et al. 2002).

Influence of 2,4-D on signal transduction and transcription factors

The results indicated that 8.4% of the genes regulated by 2,4-D are involved in cellular communication/signal transduction according to the MIPS classification. Genes that were regulated in this category mainly encoded protein kinases, serine/threonine kinases and protein phosphatases. Genes encoding the receptor protein kinase *TMK1* and serine threonine protein kinase *CTR1* were down-regulated. A possible role of the protein coded by *TMK1* in transmembrane signalling has been proposed (Chang et al. 1992). In tobacco, the accumulation of *NtTMK1* mRNA was stimulated by methyl jasmonate, wounding, fungal elicitors and other stress-related factors (Cho and Pai 2000). *CTR1* is a member of the raf histidine kinase family and a negative regulator of ethylene signal transduction (Gao et al. 2003; Huang et al. 2003).

Auxin and ABA signalling pathways are calcium dependent and calcium-dependent kinases respond to various forms of stress (Poovaiah and Reddy 1993; Yang and Poovaiah 2000). Auxin signalling is calcium/calmodulin dependent, with calmodulin known to play a significant role in auxin signalling (Poovaiah and Reddy 1993). Auxins increase cytosolic levels of calcium and the calcium-calmodulin complex, which then triggers a variety of cellular responses (Yang and Poovaiah 2000). The results of this study indicated a gene encoding a calmodulin-like protein (NaCl-inducible calcium binding) involved in signal transduction was up-regulated. There was also significant up-regulation of genes encoding NAC domain proteins involved in auxin signalling (Xie et al. 2000).

Functional classification indicated that 13.1% of the genes regulated by 2,4-D, encoded transcription factors. It was the second largest functional category to be regulated by 2,4-D. Members of the WRKY-type DNA-binding protein family and basic region/leucine zipper motif (*bZIP*) transcription factors, previously shown to be involved in pathogen defence and senescence (Eulgem et al. 2000) and stress signalling, were shown by microarray analysis to be regulated by 2,4-D. Genes encoding WRKY-type DNA-binding proteins (*WRKY4*) were down-regulated. In contrast bZIP transcription factor and ABA-responsive element-binding factor (*ABF3*) transcription factors were up-regulated, as were C3H type transcription factors (zinc finger proteins, CCCH-type, RING-H2) involved in the lignin and phenylpropanoid pathways (Franke et al. 2002). AP2/EREBP transcription factors, which play a role developmental processes and stress responses to various types of biotic and environmental stress (Riechmann and Meyerowitz 1998), were up-regulated in response to 2,4-D. *ATHB-12*, and homeobox-leucine zipper proteins (e.g. *HAT22*) of HB type transcription factors, known to be induced in response to ABA, were also up-regulated in response to 2,4-D. A C2C2 GATA-type family member (*GATA4*) was down-regulated. Members of this family are characterized by their ability to bind to the light-specific promoter with

GATA motifs (Teakle et al. 2002). Our results identified the regulation of transcription factors belonging to the AP2/ERBP, bZIP, C2C2 GATA, C3H, NAC and HB families in response to the 2,4-D treatment.

Conclusion

This is the first report of an investigation of the gene expression pattern across the whole genome of *A. thaliana* in response to the auxinic herbicide 2,4-D at a herbicidal concentration. The application of 1.0 mM 2,4-D regulated genes known to be involved in the auxin response, ethylene signalling, ABA biosynthesis, signalling and response. The results indicated that genes in both abiotic and biotic stress response detoxification (glutathione transferases, cytochrome P450s) were also modulated. The herbicide down-regulated the expression of genes involved in cell growth and elongation (*meri5B* and *TCH4*). Interestingly there was down-regulation of *sen1* and *WRKY4*, known to be induced during senescence.

The data were consistent with the pathway proposed by Hansen and Grossmann (2000). Our results indicated the 2,4-D down-regulated the expression of *ERS* and *CTR1* (negative regulators of ethylene signalling). Furthermore, there was a significant increase in expression of *AtERF4* and *AtERF8*, which may be induced in an ethylene-independent manner due to stress; these genes are also active repressors of stress response genes. The gene expression results not only confirmed that 2,4-D triggered ABA biosynthesis, as indicated by the increased expression of *NCED3*, but further indicated the regulation of genes involved downstream in ABA signalling (*ABF3*) and the ABA response.

This study importantly indicated the induction of a number of genes, either as a primary or secondary response to 2,4-D application, which have less clearly defined functions. Many of these genes have not previously been identified to have direct involvement in auxinic herbicide action. As an increased understanding of the regulation of the entire *Arabidopsis* transcriptome in response to a wider variety of chemical, physical and physiological stimuli becomes available, the exact role of these genes in response to 2,4-D should be elucidated and with this will emerge a clearer understanding of the molecular processes that occur during the plant-herbicide interactions for this widely used compound.

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