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Expression profiling of the response of *Arabidopsis thaliana* to methanol stimulation

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Abstract

In order to obtain information about the metabolism of methanol in plants, gene expression in response to methanol stimulation was analysed in leaves of *Arabidopsis thaliana* through the use of a 26,090 element oligonucleotide microarray. Following confirmation of data and the application of selection criteria, a total of 484 (1.9%) transcripts were shown to be regulated in response to a 10% methanol application. Samples were taken at 1, 24 and 72 h. Of the total identified transcripts, only 20 were shared between all three time points of which only two were down-regulated. Based on functional category analysis of these genes, at 1 h, the largest categories were metabolism, cell communication/signal transduction processes, defence genes associated with transcription and aspects of RNA processing; a few related to cell division and growth; but none were associated with photosynthesis. At 24 and 72 h, the number and overall proportion of regulated transcripts associated with metabolism increased further. Direct measurement of anthocyanin and flavonoid content confirmed that methanol-regulated transcripts corresponded to flavonoid pathways. Within the functional class metabolism, genes encoding detoxification proteins represented by far the most strongly regulated group. These included, cytochrome P450's, glucosyl transferases and members of the ABC transporter family. It is concluded that exposure to methanol affects the expression of hundreds of genes and that multiple detoxification and signalling pathways are activated.

Keywords: Gene expression profile; Microarray; Methanol; One-carbon units; Detoxification; Anthocyanins; *Arabidopsis thaliana*

1. Introduction

As one of the simplest plant products (Fall and Benson, 1996), most plants produce and emit methanol especially during early stages of development, and this volatile organic compound exits leaves via stomata (Nemecek-Marshall et al., 1995). The proportion of methanol produced that is recycled via metabolism in plants is not known, but it is clear that plant tissues can metabolise methanol (Gout et al., 2000). In plants, methanol can arise from a number of sources; for example, from pectin de-methylation in cell walls (Obendorf et al., 1990), protein repair pathways (Mudgett and Clarke, 1993), and lignin degradation (Lewis and Yamamoto, 1990). A small proportion of this endogenous methanol reaches leaf surfaces, where it is volatilised or consumed by methylotrophic bacteria (Murrell and Dalton, 1992). Exposure to exogenous methanol from

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atmospheric pollution, and deliberate application of methanol have been reported to increase growth and yield in a number of C_3, but not C_4, crops that have experienced drought stress (Nonomura and Benson, 1992a,b). However others failed to see any methanol induced growth stimulation, and the results of the field studies have proved to be largely non-reproducible (Hartz et al., 1993; Mitchell et al., 1994; Hemming et al., 1995). As a one-carbon (C1) compound, it is known that methanol in plant cells is rapidly oxidised non-enzymically to formaldehyde (Cossins, 1964; Cossins and Chen, 1997), a reactive electrophilic species with high toxicity that is rapidly detoxified. Certain reactive electrophilic species have been shown to elicit expression of defence genes in plants including genes such as chalcone synthase, dihydroflavonal reductase, allene oxide synthase, glutathione *S*-transferase and oxo-phytodienoic acid reductase (Alméras et al., 2003) and formaldehyde as the first oxidation product of methanol could act in a similar way, mediating defence signal transduction processes. The pathway for formaldehyde detoxification involves three key enzymes (Fig. 1; Gout et al., 2000; Haslam et al., 2002; Kordic et al., 2002; Achkor et al., 2003). These are: (1) NAD-dependent formaldehyde dehydrogenase (FALD), (2) the thiolesterase *S*-formylglutathione (FGH) which generates formate and (3) NAD-dependent formate dehydrogenase (FDH) which oxidises the formate to CO_2. Previously, the induction of FDH by abiotic stresses including a 20% methanol treatment has illustrated the responsive nature of this pathway (Hourton-Cabassa et al., 1998).

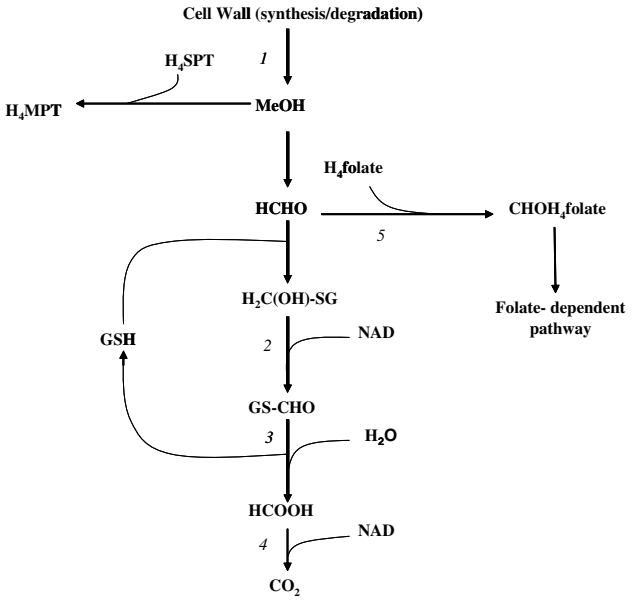

Fig. 1. An outline of the possible pathways for methanol metabolism in plants. Abbreviations used: H_4folate, tetrahydrofolate; $CHOH_4$folate, formyltetrahydrofolate; H_4SPT, tetrahydrosarcinapterin; H_4MPT, tetrahydromethanopterin. Enzymes: 1, pectin methylesterase; 2, NAD-dependent formaldehyde dehydrogenase; 3, *S*-formylglutathione hydrolase; 4, NAD-dependent formate dehydrogenase and 5, formlytetrahydrofolate synthetase.

The completion of the *Arabidopsis thaliana* genome sequencing program (Arabidopsis Genome Initiative, 2000) provides a unique resource when coupled to post-genomic technology in the form of full genome array chips, allowing the study of thousands of genes at any one time. Previously, the global response of genes to several abiotic stresses has been studied, e.g,. Seki et al. (2001, 2002) monitored expression of *Arabidopsis* genes in response to cold, drought and salt stress, whilst Fowler and Thomashaw (2002) profiled transcripts responding to cold acclimation. We use transcriptomic approaches to assess the global response of *Arabidopsis* to methanol stimulation. The present study demonstrates the impact of a chemical stimulation on networks associated with phases I–III detoxification processes (Sandermann, 1992; Coleman et al., 1997) and the anthocyanin biosynthetic pathway (Winkel-Shirley, 2002). Candidates of particular interest were validated by RNA gel blot analysis and biochemical measurements. This provided information on a range of putative methanol responsive elements, including transcriptional regulators and signalling components, for application in plant biotechnology. Characterisation of genes responding to methanol could provide an insight into gene function with regard to detoxification and stress response, which would also provide an inventory of transcripts responsive to methanol treatment. Modulation of gene expression by chemically inducible systems has many potential applications both in fundamental and applied research.

2. Results and discussion

The aim of this study was to expand our understanding of chemically regulated gene expression in plants. Specifically, the effect of methanol on gene induction and repression in *Arabidopsis* has been investigated using a microarray approach, with a view to potential agricultural and biotechnological applications. The microarray consisted of elements (>26,000) representing each of the known genes or predicted open reading frames from the *A. thaliana* genome. Foliar application of methanol resulted in the regulation of 1.9% of the genes (484 transcripts over 72 h; see Supplementary Table 1), describing a wide range of biochemical responses, many of which were associated with predicted pathways that metabolise xenobiotic compounds. The methanol treatment resulted in the wilting of leaves; however no genes were identified as being typically associated with a response to water deficit. The regulation of only 1.9% of the genes by methanol is relatively low when compared to other microarray studies (Seki et al.,

2001, 2002; Fowler and Thomashaw, 2002; Wang et al., 2003), however these represent the genes with the most significant response to methanol. Samples were taken for analysis at 1, 24 and 72 h. The threshold limit of significant change in expression level was set at $P \leqslant 0.02$ (with a %CV $\leqslant$ 30%). At the 1 and 72 h time points hybridizations were performed in triplicate, whilst the 24 h time point represents a single supplementary hybridisation. The hybridisations are evaluated in the histogram shown in Fig. 2, which indicates the cut-off values used for up- and down-regulated elements. Clearly significance will only be attached to results highlighted by analysis of the 1 and 72 h data sets; however the presence of the 24 h data set supports these observations. The interaction of genes responsive to methanol application at the three time points is illustrated by a Venn diagram in Fig. 3. The number of transcripts reached a maximum of 284 at 24 h and a minimum of 163 after 72 h. Of the total 484 identified transcripts, only 20 were shared between all three time points (detailed in Table 1), of which only two transcripts were significantly down-regulated in response to methanol, the remainder being up-regulated. In terms of the number of genes regulated, the highest number of genes shared was between the 1 and 24 h time points, and the lowest was between the 1 and 72 h time points, suggesting a logical progression of gene expression.

The supply of methanol at levels described in this study represents a xenobiotic perturbation and typically xenobiotic metabolism in plants can be divided into key stages adopted from pharmacological nomenclature (for reviews see Sandermann, 1992 and Coleman et al., 1997). Firstly, phase I reactions (activation or functionalisation) include those catalysed by the cytochrome P450 super family. Electrons from NAD(P)H are utilised to catalyse the activation of molecular oxygen leading to an oxidative attack on a range of substrates, primarily resulting in hydroxylations and (de)-methylations (Werck-Reichhart and Feyereisen, 2000). Members of the P450 family can be constitutively expressed or up-regulated by the presence of a xenobiotic (Mingot et al., 1999). Our investigations show that methanol is capable of inducing transcriptional up-regulation of a number of phase I enzymes. A total of five cytochrome P450 isoforms were regulated significantly across the time course, in addition to a putative monooxygenase (At4g15760) which may itself be a cytochrome P450. Secondly, phase II (conjugation) reactions occur. The modified hydrophilic species can be conjugated to other molecules including glutathione (electrophilic compounds), and glucose or malonic acid (compounds possessing reactive hydroxyl groups). Methanol itself contains a reactive hydroxyl and may therefore by-pass phase I routes to be conjugated immediately. However, a wide range of potential conjugating enzyme transcripts are up-regulated in response to methanol, e.g., two putative glucosyl transferases and a putative glutathione transferase were up-regulated across all three time points. Finally, phase III (sequestration) the conjugated molecules generated in phase II are tagged for transport and sequestration to cellular compartments such as the vacuole. Transport to the vacuole can occur via specific transmembrane proteins such as those belonging to the family of ABC transporters. Alternatively, modified compounds can bind to insoluble cell wall components including lignin (Langebartels et al., 1986). Across all three time points a member of the ABC (ATP binding cassette) transporter family (At2g47000) and a protein associated with exocytosis (Exo 70; At1g54090) have been flagged as methanol up-regulated elements. Other related transport proteins were up-regulated at specific time points including a number of multidrug-resistance

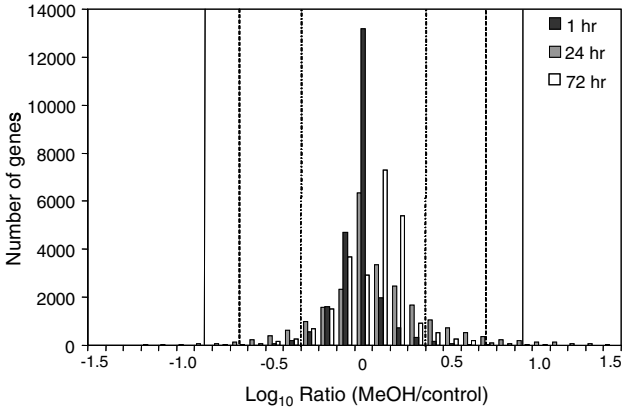

Fig. 2. Evaluation of microarray hybridisations. Histogram of $\log_{10}$ ratios of expression from methanol treated and control *Arabidopsis* plants. The values for 1 and 72 h are the average of 3 data sets, whilst the 24 h values are from one data set. The lines (dash-dot, 1 h; dash, 72 h; solid 24 h) indicate the cut-off values for up- or down-regulation.

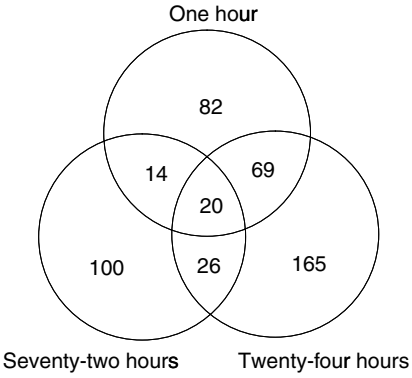

Fig. 3. Venn diagram showing intersection of genes responsive to methanol application. The number of significantly ($\geqslant$ 2-fold) up- or down-regulated transcripts shared between the three time points post methanol application are shown. Note significance can only be attached to the 1 and 72 h values whilst the 24 h values are from one data set.

Table 1
Shared transcripts significantly regulated by methanol (M) as compared to the control (C) plants across all three experimental time points

Salk annotation	AN	Log10 (M/C)		
		1 h	24 h	72 h
(1) Cell division, growth and organisation				
Expansin, putative	At2g40610.1	−0.358	−1.252	−0.863
(2) Metabolism				
Detoxification				
Putative glucosyl transferase	At2g36750.1	0.510	1.425	0.696
Putative glucosyl transferase	At2g36800.1	0.529	1.636	0.715
Glutathione transferase, putative	At1g17170.1	0.443	1.166	0.790
General				
Phenazine biosynthesis-like protein	At4g02850.1	−0.300	−0.890	−0.720
Acetyltransferase	At2g39030.1	0.337	1.006	1.135
(4) Protein synthesis and protein fate				
17.6 kDa heat shock protein (AA 1–156)	At1g53540.1	0.337	1.372	0.717
Putative RING zinc finger protein	At1g63840.1	0.592	0.951	0.705
(5) Cellular communication and signal transduction				
Protein kinases and protein phosphatases				
Protein kinase, putative	At1g26970.1	0.531	1.546	0.701
Wall-associated kinase 3	At1g21240.1	0.774	1.607	1.061
General				
Putative lectin	At3g16470.1	1.053	0.997	0.980
(6) Redox control				
Monodehydroascorbate reductase, putative	At3g09940.1	0.652	1.171	0.805
(7) Defence related				
Endochitinase isolog		0.687	1.176	0.673
Pathogenesis-related protein 1 precursor, 18.9K	At4g33710.1	0.404	1.225	0.667
Pathogenesis-related PR-1-like protein	At2g14610.1	1.496	1.192	1.129
(9) Transport				
Tol biopolymer transport system	At4g01870.1	0.641	1.355	0.679
Exo70 exocytosis	At1g54090.1	0.304	0.869	0.705
ABC transporter family protein	At2g47000.1	0.324	1.758	0.891
(10) Unknown or putative				
Mutator-like transposase, putative	At1g36105.1	0.311	1.069	0.679
Hypothetical protein	At1g21310.1	0.565	0.949	0.815

Values indicate the degrees of induction/repression calculated as ratios of the average relative intensities of treated samples.

(MDR) associated proteins and efflux pumps. It is apparent that phase I, II and III associated transcripts have been shown to respond to the methanol treatment. The response of the transcripts is complex across the time course possibly due to the fact that methanol, being a hydroxyl-containing polar molecule, is capable of being dealt with without prior phase I reactions. Additionally, the generation of the electrophilic species formaldehyde may induce a number of pathogenesis-related genes, including phase II enzymes like glutathione *S*-transferases and cytochrome P450s (Alméras et al., 2003).

Those transcripts responsive to methanol were functionally assigned to categories, as shown in Fig. 4, according to physiological function: cell division/growth, metabolism, protein synthesis and targeting, cell communication/signal transduction, reduction–oxidation processes, defence related, transcription/RNA processing and transport. Remaining transcripts with no clearly ascertainable role were labelled as unclassified or unknown. After 1 h, a total of 184 transcripts were shown to be significantly regulated and of these 114 represented genes either of known function or to which function could be assigned putatively on the basis of homology. The largest represented functional classes at the 1 h time point were metabolism (30 genes), genes representing cell communication and signal transduction processes (24 genes) and defence associated genes

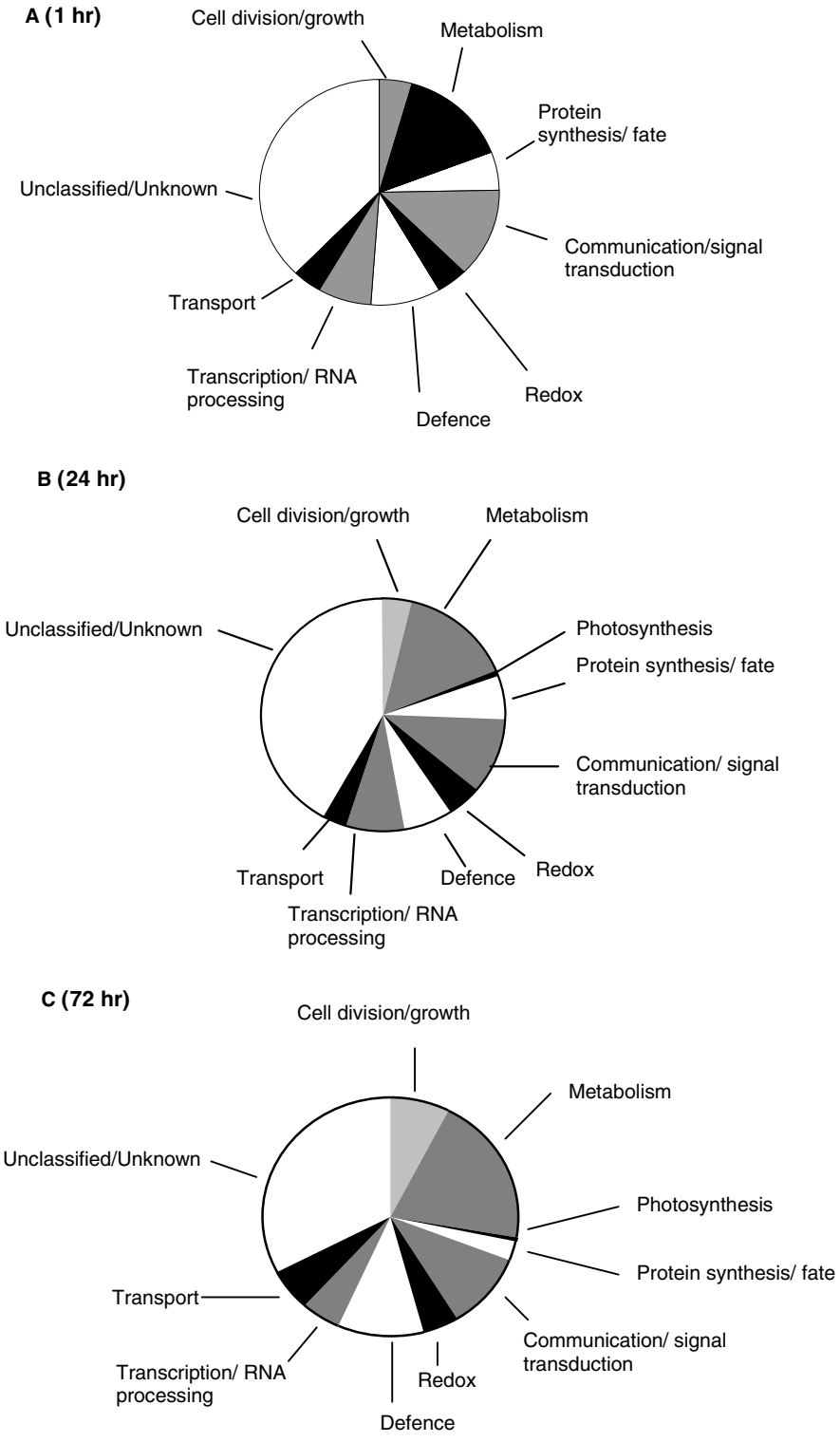

Fig. 4. Functional categorisation of responsive transcripts. Charts illustrating the functional physiological categorisation of the identified 476 methanol regulated transcripts, where A, 1 h; B, 24 h; and C, 72 h in which data was collected from three slide hybridisations at 1 and 72 h, and one hybridisation at 24 h.

(19). Additionally, genes associated with transcription and aspects of RNA processing were well represented (14 genes) and these included a wide spectrum of transcription factors or DNA binding proteins principally of the bZIP, MYB, RING zinc finger and WRKY classes. A number of genes within cell division and

growth were responsive, particularly the down-regulation of an expansin. It was apparent that few genes related to photosynthesis were regulated by methanol. Finally, there was a steady increase across the time course in the number of regulated elements encoding products associated with amino acid metabolism and protein synthesis, e.g., transaminases and endopeptidases. The general pattern of gene expression for 1 h post methanol application is repeated for the 24 and 72 h time points (see Fig. 4).

The metabolism and communication/signal transduction categories were sub-divided to enable more detailed analysis of the response in these groups (Fig. 5). Of the genes responding to methanol many encoded detoxification proteins from a wide range of xenobiotic metabolising pathways, examples including cytochrome P450s, glucosyl transferases and members of the ABC transporter family (see Supplemental Table 1). Transcripts corresponding to flavonoid pathways were represented at the 24 and 72 h time points. Putative genes encoding key enzymes in the anthocyanin biosynthetic pathway, namely dihydroflavanol reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX) are induced at 24 and 72 h, whereas genes encoding other flavonoid biosynthetic enzymes, e.g., flavonol synthase (FLS) are down-regulated. In addition to wilting the most visible outcome of methanol spraying was the production of anthocyanin in treated plants. Measurement of flavonoid and anthocyanin pigment levels in methanol treated leaf tissue (Table 2), showed an increase of greater than 2-fold anthocyanin content and a small increase in total flavonoid content over the 72 h of methanol exposure. The physical appearance of the sprayed plants was also an indication of the accumulation of anthocyanins within leaf tissue (Fig. 7, top right panel). The flavonoids are a ubiquitous group of stress-related secondary metabolites (Winkel-Shirley, 2002). The microarray data indicated that methanol affected a major branch point of the flavonoid pathway, specifically a deflection of pathway flux towards flavan-3,4-diol and anthocyanin biosynthesis, and away from flavonol biosynthesis. This result was evident from both transcript changes and direct measurements of anthocyanin and flavonoid levels in leaf extracts. It has been suggested that flavonols, being the most ancient and taxonomically widespread flavonoid are the most important in terms of stress responses (Stafford, 1991; Morant et al., 2003), yet methanol apparently down-regulated the key biosynthetic enzyme flavonol synthase. However, anthocyanins have also been well documented in terms of their protective function and have been reported to have a particular role in the protection of leaf cells from photo-oxidative damage (Chalker-Scott, 1999; Neill and Gould, 2003), possibly improving the efficiency of nutrient retrieval during senescence or similar developmental processes (Field et al., 2001). It is possible that transporters identified here are involved in secondary metabolite pathways such as anthocyanin transport, rather than methanol metabolite efflux. Further, the high representation of glycosyl transferase-like transcripts points to an involvement in glycosylation of the increased pool of flavonoid molecules including the anthocyaninidins. Even the well-documented conjugating enzymes, the glutathione *S*-transferases, are known to have diverse roles, for example AN9 in Petunia has been found to encode a glutathione *S*-transferase that

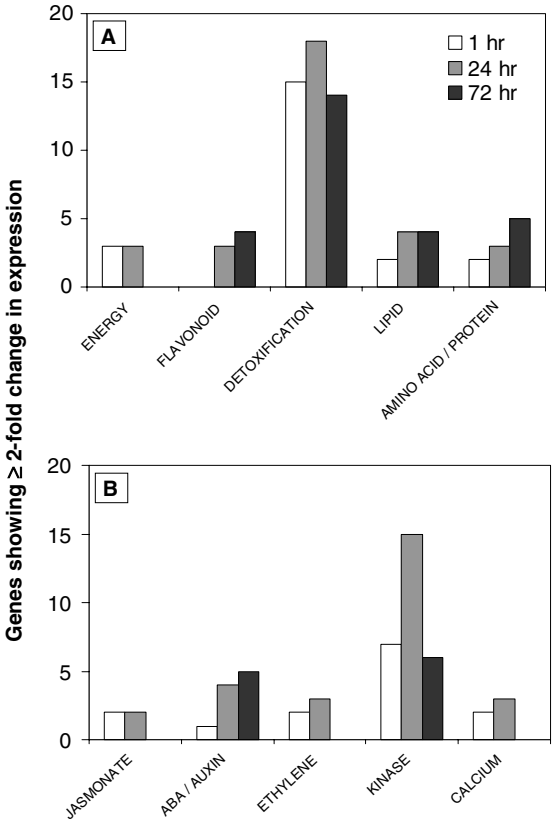

Fig. 5. A breakdown on the basis of specific pathway or function of genes identified as being significantly (note significance can only be attached to the 1 and 72 h values whilst the 24 h values are from one data set) regulated in the categories of metabolism (A) and communication/signal transduction (B) over the time course of methanol treatment. This analysis allows each gene to be allocated to only one sub-category based on a specific pathway or function.

Table 2
Anthocyanin and total flavonoid content in leaf tissue during time course of methanol application

Time (h)	Anthocyanin (A300 gfwt^{-1})	Flavonoid (A530 gfwt $^{-1}$)
0	4.425 ± 0.182	0.916 ± 0.057
1	5.034 ± 0.239	1.066 ± 0.090
8	6.420 ± 0.234	1.043 ± 0.080
24	7.361 ± 0.448	1.051 ± 0.081
48	9.743 ± 0.508	1.172 ± 0.107
72	10.175 ± 0.500	1.366 ± 0.120

Each value represents the mean of five separate extracts, with standard errors as indicated.

is a flavonoid binding protein with a role in anthocyanin sequestration (Mueller et al., 2000).

Shown in Fig. 5B are the major intracellular signalling sub-categories suggested from the array data to be significantly regulated by methanol stimulation. Notably, kinases represent the largest group of methanol responsive elements across all three time points (all up-regulated with the exception of putative NPK1-related protein kinase). A range of transcripts associated with downstream signalling and activation processes were identified within this sub-category, including MAP kinases and receptor kinases. Analysis revealed a progressive increase in the number of ABA- and auxin-related transcripts over the three time points, e.g., aldehyde oxidase. Transcripts putatively encoding jasmonate signalling pathway genes, e.g., oxophytodienoic acid reductase-1 (OPR1) were significantly up-regulated at 1 h (and 24 h), but not after 72 h. A pattern of increased expression was seen for ethylene signalling (e.g., the ethylene biosynthetic enzyme 1-aminocyclopropane-1-carboxylate oxidase (ACO)) and calcium signalling (e.g., calmodulin-like protein) sub-categories.

The 20 significantly regulated elements common to all three time points are listed in Table 1. With the exception of photosynthesis and transcription factors, all major functional categories are represented, with detoxification metabolism and defence being the largest groups. The three detoxification genes all putatively encode phase II metabolic enzymes, i.e., conjugation to either sugars or glutathione mediated by glucosyl transferases and glutathione *S*-transferases. Defence genes within the list include those for PR1-like proteins and precursors. Transport genes encoding probable phase III proteins, e.g., an ABC family transporter, are also up-regulated. Only two of the elements listed are down-regulated, namely a putative expansin within the cell division and growth category, and a phenazine biosynthesis-like gene within general metabolism. Expansins have been identified as cell-wall-loosening proteins that mediate extension of the plant cell wall (Cosgrove, 2000). Down-regulation of growth promoting agents such as these suggests a re-direction of resources to defence and essential metabolic functions. The possible role of the extracellular matrix in signalling the presence of methanol is suggested by the up-regulation of a cell wall associated kinase (WAK: At1g21250.1). Induced by pathogen infection and wounding these proteins span the plasma membrane and extend into the cell wall (Andersen et al., 2001).

The transcript abundance of a selected range of genes whose expression was altered significantly in the microarray experiment was analysed by RNA gel blot analysis (Fig. 6). The analysis was done using gene specific primers to generate probes corresponding to the following genes: pathogenesis-related protein 1 (PR1; At2g14610), oxo-phytodienoic acid reductase 1 (OPR1; At1g76680), putative glutathione *S*-transferase (GST; At1g17170), putative cytochrome P450 (CYT; At1g64930), putative monodehydroascorbate reductase (DHAR; At3g09940), and a putative expansin (EXP; At2g40610). As illustrated (Fig. 6) PR1, OPR1, GST and CYT all showed an increase in expression over the time course, strongly supporting the microarray data. Although DHAR increased at 1 h but then showed a reduction in expression at 24 and 72 h, whilst the array analysis indicated an induction in transcript abundance at 1 and 24 h (see Supplementary Table 1). Analysis of the putative expansin confirmed a down-regulation of this protein over the 72 h time course. The best-understood metabolic route of methanol involves its non-enzymatic oxidation to the reactive electrophile formaldehyde (Cossins, 1964; Gout et al., 2000; Hourton-Cabassa et al., 1998; Hanson and Roje, 2001). Two genes documented as part of the folate-independent C1 pathway were selected for validation by RNA

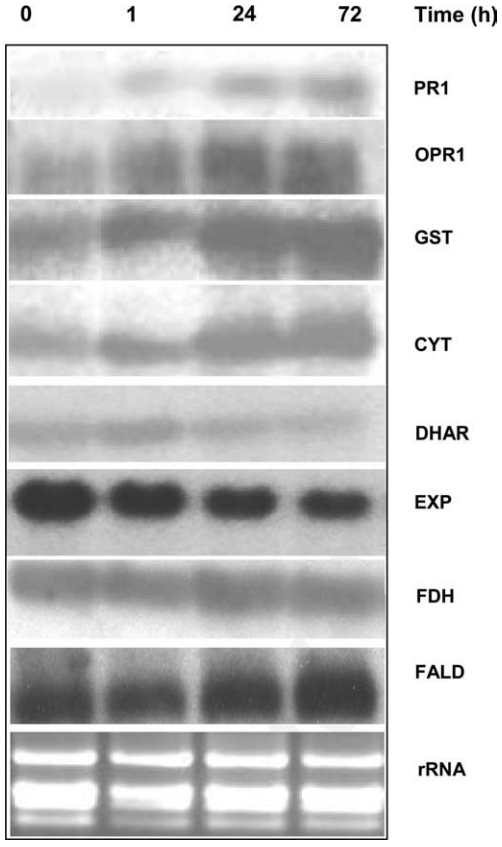

Fig. 6. RNA gel blot analysis illustrating the response of individual transcripts over the time course of Methanol application. Total RNA was extracted from leaves of *A. thaliana* 1, 24 and 72 h post methanol spraying, and probed with cDNA corresponding to transcripts of interest. (PR1, pathogenesis related protein-1; OPR1, oxophytodienoate reductase-1; GST, putative glutathione *S*-transferase, CYT, cytochrome P450; DHAR, dehydroascorbate reductase. EXP, expansin, FDH, NAD-dependent formate dehydrogenase; FALD, glutathione-dependent formaldehyde dehydrogenase).

gel blot analysis. The selected genes were NAD-dependent formate dehydrogenase (FDH; At5g14780) known to have an important role in formate tolerance (Li et al., 2002) and glutathione-dependent formaldehyde dehydrogenase (FALD; At5g43940) as it plays an important role in cell protection in response to stress as well as its defined role in formaldehyde metabolism (Sakamoto et al., 2002; Diaz et al., 2003). The blots showed no evidence of up- or down-regulation of these transcripts over the time course, confirming the conclusion from the microarray data that this part of the C1 pathway did not respond to the methanol treatment (Fig. 6). Previous studies have investigated the effects of foliar methanol application on growth and productivity of crop species, with diverse conclusions. Nonomura and Benson (1992a) reported an increase in yield of a variety of C_3 crop species when sprayed with 10–50% methanol solutions, which was obvious only when the plants had experienced some water deficit. Other workers have failed to observe these growth enhancing effects (Hemming et al., 1995; Iersel et al., 1995; Rajala et al., 1998). The results presented here suggest that methanol, supplied at the rates described here, has a suppressive effect on plant growth. Alternative pathways for methanol utilisation observed in other evolutionary groups including the *Archea*, involving the novel cofactor tetrahydrosarcinapterin, which in *Methanoarchaea* can produce the methylated derivative via direct reaction with methanol, or via the intermediary of methylated coenzyme M (Ferry, 1999). Seven putative genes from this pathway, encoding methyltransferases, have been identified in *Arabidopsis*, (*TAIR*: www.arabidopsis.org). One of these genes (At1g78240) was found to have a 1.6-fold change in expression at 1 h and a 2.1-fold change at 24 h according to our data. Although these changes are below the selection thresholds based on our criteria, the pathway may present one part of a total capacity for dealing with methanol.

The perturbation that accompanies methanol application generated a surprisingly large and complex increase in transcriptional activity. The majority of these transcription factors are up-regulated after 1 h suggesting an initial burst of transcriptional activity prior to synthesis of methanol-responsive proteins. Well-known categories of transcription factors (TFs) (Ellenberger, 1994; Stracke et al., 2001) have been shown to be methanol-responsive, including basic region/leucine zipper (bZIP) proteins, members of the WRKY class and MYB proteins. Blast analysis showed the methanol responsive WRKY TFs to be similar to those identified in rice. *Cis*-regulatory elements and interacting *trans*-acting factors have a number of complex and as yet incompletely understood functions, and many TFs are themselves subject to complex regulation (HannaRose and Hansen, 1996). Interaction with, and regulation of, the phenylpropanoid pathway genes in plants is a notable role for some of the characterised TFs highlighted by the microarray data as being important in methanol stimulation (for example, see induction of anthocyanin biosynthetic genes in maize by a MYB and a bHLH protein; Perrot and Cone, 1989). BLAST analysis of the methanol responsive MYB TFs identified an up-regulated MYB family transcription factor (At1g66380) that showed a 75% identity to *anthocyanin 1* (ANT1). ANT1 is known to cause the up-regulation of genes that encode proteins in both the early and later steps of anthocyanidin biosynthesis as well as genes involved in the glycosylation and transport of anthocyanins into the vacuole (Mathews et al., 2003).

Presented in this study are the first steps to identifying those genes responsive to methanol. It is difficult to ascertain whether we are observing a general toxicity response, and/or the induction by methanol or one of its metabolites mediating these effects. A range of electrophilic species such as unsaturated carbonyl compounds have been shown to elicit many of the stress responses described in this study (Alméras et al., 2003), so the possibility of the oxidation product, formaldehyde, being the active electrophilic elicitor cannot be ruled out. Future experiments might consider the application of formaldehyde and/or other alcohols. The application of methanol as a candidate for gene induction in plants is clear, already ethanol has been used successfully to manipulate carbon metabolism in plants (Caddick et al., 1998). Although the use of endogenous genes may carry a basal level of activity and incur possible developmental effects. This can be offset by the convenient application, low cost and induction of a relatively small subset of genes. Indeed some of the transcript changes identified could be developed in the future as sensitive bio-indicators of environmental pollution. Obtaining chemically inducible promoters would be a real breakthrough for agricultural biotechnology as progress in this area is limited (Gatz and Lenk, 1998).

3. Experimental procedures

3.1. Plant growth conditions and methanol treatment

Arabidopsis thaliana cv Columbia wild type plants were grown in controlled conditions (10 h, 25 °C day/14 h, 18 °C night, 70% relative humidity, supplied with a instantaneous photosynthetic photon flux density of 300 $\mu mol\, m^{-2} s^{-1}$ over a 12 h photoperiod). Twenty pots containing two five-week-old plants each were sprayed with a 10% (v/v) methanol solution containing 0.1% (v/v) Silwet® surfactant (control plants were sprayed with water and Silwet®) at a rate of 248 $L\, ha^{-1}$ or 100 mL across the 20 pots, using an automated Track sprayer unit fitted with a Teejet nozzle (TJ110015VK). Two blanket sprays were applied, five minutes apart,

with the nozzle approximately 50 cm above the leaf surface. A concentration of 10% (v/v) methanol was used in order to expose plants to a concentration of methanol in excess of endogenous levels. Subsequently, to maintain this level each pot of plants was sprayed after 24 and 72 h with ~10 mL methanol/Silwet® using a hand held sprayer, whilst control plants were sprayed with water and Silwet®. In order to prevent any methanol contamination pots were removed from the chamber, sprayed, and immediately replaced.

3.2. Pigment analysis

Water-soluble pigments (flavonoids and anthocyanins) were extracted from leaves at the end of the drought treatment using the method of Jordan et al. (1994). Leaves from five separate plants per time point (0.5 g per sample) were ground to a powder in liquid nitrogen before extraction in 10 mL of acidified methanol (HCl:methanol, 1:99, v/v). Absorption spectra of the extracts were determined using a Cary 210 spectrophotometer (Varian, Palo Alto, CA), and the flavonoid and anthocyanin contents were estimated from absorbance's at 300 and 530 nm, respectively.

3.3. Isolation of RNA and RT-PCR amplification

Total RNA was prepared from green leaf tissue removed from control plants and methanol-treated plants (samples taken at 1, 24 and 72 h post methanol application in an attempt monitor the phases of detoxification). One leaf was taken per plant from each pot and immediately frozen in liquid nitrogen. The fresh tissue was then rapidly ground in liquid nitrogen and 1.5 mL Trizol™ reagent (Life Technologies) added to 200 mg of ground material. The Trizol™ extraction method was then followed according to the manufacturers' instructions. Total RNA (1 μg) was used for RT-PCR amplification of transcripts of interest using the Promega Access RT-PCR system according to the manufacturers' instructions. An annealing temperature of 60 °C for 1 min and a 2 min extension at 68 °C was used over 35 cycles. Gene specific forward (F) and reverse (R) primers were used as follows: Pathogenesis related protein-1 (PR1) (F) 5′-TTTACTG- GCTATTCTCGATTTTTA-3′, (R) 5′-CCCACGAGGATCATAGTT-3′; Oxophytodienoate reductase-1 (OPR1) (F) 5′-GC CTCACGCT-GCCATATATTACTC-3′, (R) 5′-GTCTGGTCC GAT CTC C T- TAGCAAC-3′; Putative glucosyl transferase (GT) (F) 5′-CCAATGT TGA TGA T TCTTCTCA-GCCTT-3′ (R) 5′-GGCC GTGTCTCCCATGTAG-CG-3′; Putative cytochrome P450 (CYT) (F) 5′-TCAT CAAAC AAAGACAATAATGGAAAC-3′ (R) 5′-TA-CTT CTGG ATG G TTCAACAAATT-3′; Dehydroascorbate reductase (DHAR) (F) 5′-GCGG AAGA GA AAA GCTACAAG-3′ (R) 5′-GGTG CTA AAT CCAGTTGCTAC-3′; putative expansin (EXP) (F) 5′-CGC AAGT GCCT GCTGGTTATTTTATTT-3′ (R) 5′-GA- CGG GGA TACCAGCCTT-3′; NAD-dependent formate dehydrogenase (FDH) (F) 5′-AGG AGTTTTCTACAAGGCCAA-3′ (R) 5′-CAACATG-TCTTTCGTCCCCGC-3′; Glutathione-dependent formaldehyde dehydrogenase (FALD) (F) 5′-CCTTGTA-TTCTAGGTCATGAGGCTGCT-3′ (R) 5′-CAAGTT-GTCTGTGTTATGTATTCGTCC-3′. RT-PCR products were purified using the Wizard Direct DNA purification system (Promega).

3.4. RNA gel blot analysis

Total RNA (10 μg per sample) was loaded on 1.2% agarose formaldehyde gels and blotted to nylon membrane (Hybond NX, Amersham) by capillary action in 20 × SSC (175.3 g/L NaCl, 88.2 g/L trisodium citrate, pH 7.0; Sambrook et al., 1989). The air dried membrane was then cross linked by UV irradiation in a Stratalinker™ 1800 (Stratagene). Purified cDNA's corresponding to genes of interest were labelled directly with [α-^{32}P]dCTP using Prime-a-gene labelling system (Promega) as per the manufacturers' instructions. Probes were purified using a Nuctrap® (Stratagene) column. Labelled probes were allowed to hybridize for a minimum of 12 h to membrane cross-linked RNA equilibrated in buffer (200 mM sodium phosphate, pH 7.2, 6.6% (w/v) SDS, 1 mM EDTA 1% BSA/cm^2 membrane) at 65 °C. Membranes were then washed twice for 10 min in 2 × SSC, 0.1% (v/v) SDS at 65 °C and autoradiographed between intensifying screens at −80 °C.

3.5. Expression profiling

In order to remove any possible effects of diurnal or circadian rhythms (Harmer et al., 2000), samples of tissue from control/stress plants were harvested contemporaneously at identical time points. In addition, to account for differences between plants each sample consisted of leaf tissue of the same developmental stage taken from six separate plants. Poly(A)$^+$ RNA was isolated from 600 μg of total RNA (polyATract mRNA isolation kit; Promega, WI) and for each time point replicates were labelled by incorporation of Cy3/Cy5 fluorescent dyes. Hybridisation and probe preparation was performed according to (Wang et al., 2003), where Poly(A)$^+$ RNA was mixed with 0.6 mM each of dATP, dCTP, dGTP, 0.5 mM dTTP, 0.3 mM 5-(3-aminoallyl)-2′deoxyuridine-5′triphosphate (Sigma), 0.03 mM of either Cy3-dUTP or Cy5-dUTP dye ester (Amersham Pharmacia), 2 lg oligo(dT) (Invitrogen) and then first strand cDNA was made with Superscript II RT (Invitrogen). Microfarad slides (printed in the laboratory of Dr. David Galbraith, University of Arizona, USA) were

used, consisting of The Arabidopsis Genome Oligo Set Version 1.0 containing 26,090 70 oligomers (Qiagen/Operon, Inc.) representing 26,090 genes and predicted open reading frames obtained from the UniGene Database Build At 4, developed at the National Centre of Biotechnology Information (www.ncbi.nlm.gov/UniGene). The Qiagen Operon Microaray Database (OMAD; www.operon.com/arrays/omad.php) provides information on each probe and the gene it represents. After incubation for 2 h at 42 °C, the cDNA was treated with 2 units of RNaseH (Invitrogen) for 15 min at 37 °C. The buffer system was modified to do this step. After the binding of cDNA with PB buffer (supplemented), the column was washed with phosphate–EtOH buffer (5 mM KPO_4 pH 8.0, 80% EtOH) and the cDNA was eluted with phosphate buffer (4 mM KPO_4 pH 8.5) instead of the kit supplemental wash and elution buffer. Among the replicate experiments within each time point, the Cy3 and Cy5 labels were swapped between sample and control cDNA to minimise any possible impact of inequalities in DNA incorporation and photo-bleaching of the fluorescent dyes. The labelled cDNA was purified with Qiagen PCR purification system. All supplemental buffers were used following the manufacturers' instructions. After purification, the labelled-cDNA was dried. Cy3 and Cy5-labelled cDNA were combined and then dissolved in 82 lL hybridisation buffer (25% formamide, 5 × SSC 0.1% SDS, 1 mM DTT, 100 lg/mL sonicated salmon sperm DNA). Hybridisation was performed in high-humidity chambers (42 °C; 20 h). The slides were washed successively in 2 × SSC, 0.1% SDS, then 0.1 × SSC, 0.1% SDS, 0.1 × SSC for 5 min each step at 42 °C. Three hybridisations were prepared for the 1 and 72 h time points respectively, representing three biological repetitions. A supplementary single hybridisation was prepared for the 24 h time point. The overall strategy employed in the study of methanol-regulated gene expression by microarray analysis is summarised in Fig. 7.

3.6. Data acquisition and analyses

Slides were scanned and spot intensities were extracted (Genepix 4000 scanner; GenePix Pro 3.0; Axon Instruments, CA). The intensity of all spots on the filter was averaged and total fluorescence normalization was adopted for normalising the difference of signal intensity between replicate arrays. An average background value was subtracted from each of the spot intensities, which was then divided by the average intensity value of the sum of all the spot intensities. Fluorescence intensity ratios (Cy5/Cy3) were obtained from all target elements. All ratio values were log transformed (base 10) to treat inductions or repressions of identical magnitude as numerically equal but of opposite sign. In some cases, individual spots were flagged for rejection on the basis

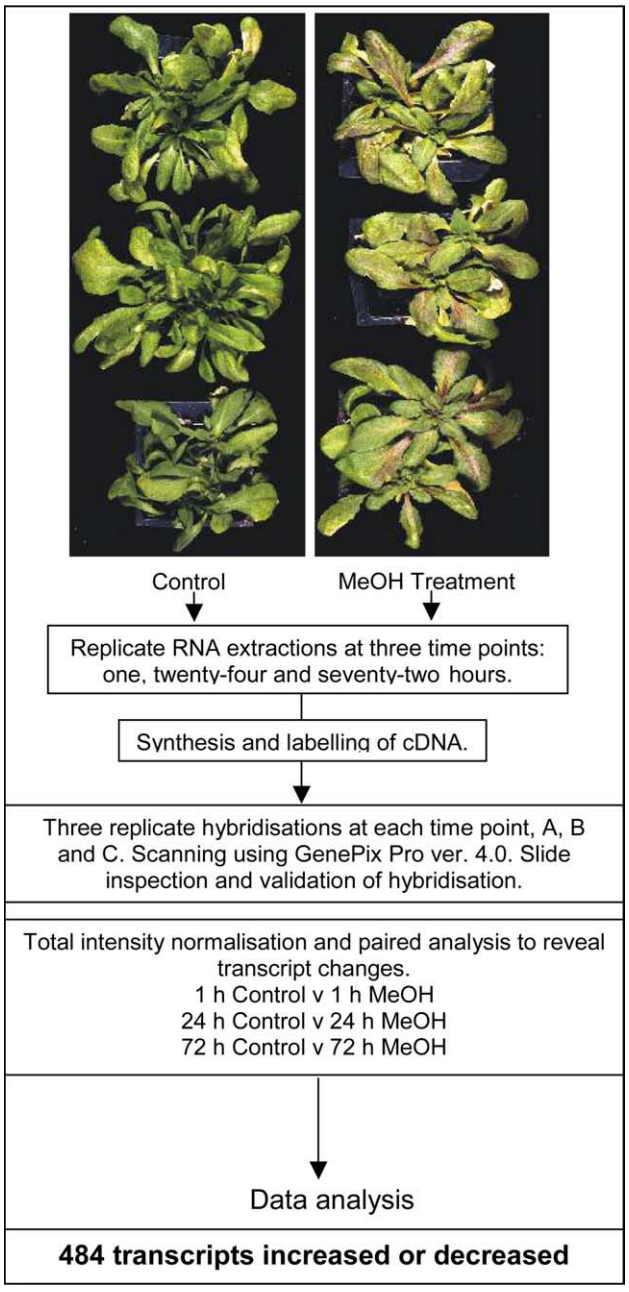

Fig. 7. Strategy for cDNA microarray analysis of methanol responsive transcripts. Images of control and treated *Arabidopsis* plants are shown at the end of the methanol treatment.

of poor hybridization as determined by the image analysis software in conjunction with visual inspection. Data were averaged and analysed using Microsoft Excel and Spotfire software (http://www.spotfire.com/). Averaged data points with fluorescence intensity ratio greater than a minimum threshold value were considered as up- or down-regulated at the 1 and 72 h time points, respectively. The threshold levels applied were based on significance testing by application of a two-tailed *t*-test (Welch's *t*-test) which assumes unequal variance between data sets and a coefficient of variation

(%CV) $\leqslant$ 30%. The data filtering retained the top 1.5% of the up- and down-regulated elements with respect to the control position, $P \leqslant 0.02$, as illustrated in Fig. 2. Application of this significance criterion to the single hybridization at 24 h translated to a minimum 7.5-fold change in expression level. The functional classification of genes showing regulated expression in response to methanol treatment was based on the functional organisation of the *Arabidopsis* genome (http://mips.gsf.de/proj/thal/db/index.html) and accession number based database interrogation using Expasy (http://ca.expasy.org/), BLAST sequence analysis (http://www.ncbi.nlm.nih.gov/BLAST/) and the NCBI protein domain search tool (http://www.ncbi.nlm.nih.gov).

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Appendix A. Supplementary material

Table S1. All transcripts significantly regulated by methanol at the experimental time points. Values indicate the degrees of induction/repression calculated as ratios of the average relative intensities of treated samples. Values in red and green represent statistically significant induction and repression, respectively ($P = 0.02$, %CV = 30) for the 1 and 72 h time points. The results from a single slide hybridisation at 24 h are shown. Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.phytochem.2004.07.006.

References

Achkor, H., Diaz, M., Fernandez, M.R., Biosca, J.A., Pares, X., Martinez, M.C., 2003. Enhanced formaldehyde detoxification by overexpression of glutathione-dependent formaldehyde dehydrogenase from *Arabidopsis*. Plant Physiol. 132 (4), 2248–2255.

Alméras, E., Stolz, S., Vollenweider, S., Reymond, P., Mène-Saffrané, L., Farmer, E.E., 2003. Reactive electrophile species activate defence gene expression in *Arabidopsis*. Plant J. 34, 205–216.

Andersen, C.M., Wagner, T.A., Perret, M., He, Z.H., He, D., Kohorn, D., 2001. WAKs: cell wall-associated kinases linking the cytoplasm to the extracellular matrix. Plant Mol. Biol. 47, 197–204.

Arabidopsis Genome Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature (London) 408, 796–815.

Caddick, M.X., Greenland, A.J., Jepson, I., Krause, K.P., Qu, N., Riddell, K.V., Salter, M.G., Schuch, W., Sonnewald, U., Tomsett, A.B., 1998. An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nat. Biotechnol. 16, 177–180.

Chalker-Scott, L., 1999. Environmental significance of anthocyanins in plant stress response. Photochem. Photobiol. 70, 1–9.

Coleman, J.O.D., Blake-Kalff, M.A., Davies, E.T.G., 1997. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. Trends Plant Sci. 2 (4), 144–151.

Cosgrove, D.J., 2000. Expansive growth of plant cell walls. Plant Physiol. Biochem. 38, 109–124.

Cossins, E.A., 1964. The utilization of carbon-1 compounds by plants. I. The metabolism of methanol-^{14}C and its role in amino acid biosynthesis. Can. J. Biochem. 42, 1793–1802.

Cossins, E.A., Chen, L.F., 1997. Folates and one-carbon metabolism in plants and fungi. Phytochemistry 45 (3), 437–452.

Diaz, M., Achkor, H., Titarenko, E., Martinez, M.Z., 2003. The gene encoding glutathione-dependent formaldehyde dehydrogenase/GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid. FEBS Lett. 543 (1–3), 136–139.

Ellenberger, T., 1994. Getting a grip on DNA recognition: structures of the basic region leucine zipper, and the basic region helix-loop-helix DNA-binding domains. Curr. Opin. Struct. Biol. 4, 12–21.

Fall, R., Benson, A.A., 1996. Leaf methanol – the simplest natural product from plants. Trends Plant Sci. 9, 296–301.

Ferry, G., 1999. Enzymology of one-carbon metabolism in methanogenic pathways. FEMS Microbiol. Rev. 23 (1), 13–38.

Field, T.S., Lee, D.W., Holbrook, N.M., 2001. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol. 127, 566–574.

Fowler, S., Thomashaw, M.F., 2002. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. The Plant Cell 14, 1675–1690.

Gatz, C., Lenk, I., 1998. Promoters that respond to chemical inducers. Trends Plant Sci. 3 (9), 352–358.

Gout, E., Aubert, S., Bligny, R., Rebeille, F., Nonomura, A., Benson, A., Douce, R., 2000. Metabolism of methanol in plant cells. Carbon-13 nuclear magnetic resonance studies. Plant Physiol. 123, 287–296.

HannaRose, W., Hansen, U., 1996. Active repression mechanisms of eukaryotic repressors. Trends Gen. 12, 229–234.

Hanson, A.D., Roje, S., 2001. One-carbon metabolism in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 119–137.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., Kay, S.A., 2000. Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290, 2110–2113.

Hartz, T., Mayberry, K.S., McGiffen, M.E., LeStrange, M., Miyao, E., 1993. Foliar methanol application ineffective on tomato and melon. Unpublished report from the Department of Vegetable Crops, University of California, Davis, CA.

Haslam, R., Rust, S., Pallett, K., Cole, D., Coleman, J., 2002. Cloning and characterisation of *S*-formylglutathione hydrolase from *Arabidopsis thaliana*: a pathway for formaldehyde detoxification. Plant Physiol. Biochem. 40, 281–288.

Hemming, D.J.B., Criddle, R.S., Hansen, L.D., 1995. Effects of methanol on plant respiration. J. Plant Physiol. 146, 193–198.

Hourton-Cabassa, C., Ambard-Bretteville, F., Moreau, F., Davy de Virville, J., Rémy, R., Colas des Francs-Small, C., 1998. Stress induction of mitochondrial formate dehydrogenase in potato leaves. Plant Physiol. 116, 627–635.

Iersel, M., Heitholt, J., Wells, R., Oosterhuis, M., 1995. Foliar methanol applications to cotton in the south eastern United States: leaf physiology, growth and yield components. Agron. J. 87, 1157–1160.

Jordan, B.R., James, P.E., Strid, A., Anthony, R.G., 1994. The effect of ultraviolet-B radiation on gene expression and pigment composition in etiolated and green pea leaf tissue: UV-B induced changes are gene-specific and dependent upon the development stage. Plant Cell Environ. 17, 45–54.

Kordic, S., Cummins, I., Edwards, R., 2002. Cloning and characterization of S-formylglutathione hydrolase from *Arabidopsis thaliana*. Arch. Biochem. Biophys. 399 (2), 232–238.

Langebartels, C., Harms, H., Arjmand, M., Schäfer, W., Sanderman, H., 1986. Cell wall components as binding sites for xenobiotics. In: Vain, B., Reis, D., Goldberg, R. (Eds.), Cell Walls '86. Université Pierre et Marie Curie, Paris.

Lewis, N.G., Yamamoto, E., 1990. Lignin: occurrence, biogenesis and biodegradation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 455–496.

Li, R., Moore, M., Bonham-Smith, P.C., King, J., 2002. Overexpression of formate dehydrogenase in *Arabidopsis thaliana* resulted in plants tolerant to high concentrations of formate. J. Plant Physiol. 159 (10), 1069–1076.

Mathews, H., Clendennen, S.K., Caldwell, C.G., Liu, X.L., Connors, K., Matheis, N., Schuster, D.K., Menasco, D.J., Wagoner, W., Lightner, J., Wagner, D.R., 2003. Activation tagging in tomato identifies a transcriptional regulator of anthcyanin biosynthesis, modification, and transport. Plant Cell 15, 1689–1703.

Mingot, J.M., Penalva, M.A., Fernandez-Canon, J.M., 1999. Disruption of *phacA* an *Aspergillus nidulans* gene encoding a novel cytochrome P450 monooxygenase catalyzing phenylacetate 2-hydroxylation results in penicillin overproduction. J. Biol. Chem. 274, 14545–14550.

Mitchell, A.R., Crowe, F.J., Butler, M.D., 1994. Plant performance and water use of peppermint treated with methanol and glycine. J. Plant Nutrit. 17, 1955–1962.

Morant, M., Bak, S., Lindberg Møoller, B., Werck-Reichhart, D., 2003. Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. Curr. Opin. Biotechnol. 14, 151–162.

Mudgett, M.B., Clarke, S., 1993. Characterisation of plant L-isoaspartyl methyltransferases that may be involved in seed survival: purification, cloning and sequence analysis of the wheat germ enzyme. Biochemistry 32, 11100–11111.

Mueller, L.A., Goodman, C.D., Silady, A., Walbot, V., 2000. AN9, a Petunia glutathione *S*-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. Plant Physiol. 123, 1561–1570.

Murrell, J.C., Dalton, H. (Eds.), 1992. Methane and Methanol Utilizers. Plenum Press.

Nemecek-Marshall, M., Macdonald, R.C., Franzen, J.J., Wojciechowski, C.L., Fall, R., 1995. Methanol emission from leaves: enzymatic detection of gas phase methanol and relation of methanol fluxes to stomatal conductance and leaf development. Plant Physiol. 108, 1359–1368.

Neill, S.O., Gould, K.S., 2003. Anthocyanins in leaves: light attenuators or antioxidants. Func. Plant Biol. 30, 865–873.

Nonomura, A.M., Benson, A.A., 1992a. The path of carbon in photosynthesis: improved crop yields with methanol. Proc. Natl. Acad. Sci. USA 89, 9794–9798.

Nonomura, A.M., Benson, A.A., 1992. The path of carbon in photosynthesis: methanol and light. In: Murata, N. (Ed.), Research in Photosynthesis, vol. 3, pp. 911–914.

Obendorf, R.L., Koch, J.L., Gorecki, R.J., 1990. Methanol accumulation in maturing seeds. J. Exp. Bot. 41, 489–495.

Perrot, G.H., Cone, K.C., 1989. Nucleotide sequence of the maize R-S gene. Nucleic Acids Res. 17, 8003.

Rajala, A., Kärkkäinen, J., Peltonen, J., Peltonen-Sainio, P., 1998. Foliar applications of alcohols failed to enhance growth and yield of C3 crops. Ind. Crops Prod. 7, 129–137.

Sakamoto, A., Ueda, M., Morikawa, H., 2002. Arabidopsis glutathione-dependent formaldehyde dehydrogenase is an *S*-nitrosoglutathione reductase. FEBS Lett. 515 (1–3), 20–24.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press.

Sandermann, H., 1992. Plant metabolism of xenobiotics. Trends Biochem. Sci. 17, 82–84.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., Shinozaki, K., 2001. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. The Plant Cell 13, 61–72.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., 2002. Monitoring the expression profiles of 7,000 *Arabidopsis* genes under drought, cold and high salinity stresses using a full-length cDNA microarray. Plant J. 31, 279–292.

Stafford, H.A., 1991. Flavonoid evolution: an enzymatic approach. Plant Physiol. 96, 680–685.

Stracke, R., Werber, M., Weisshaar, B., 2001. The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr. Opin. Plant Biol. 4, 447–456.

Wang, H., Miyazaki, S., Kawai, K., Deyholos, M., Galbraith, D.W., Bohnert, H.J., 2003. Temporal progression of gene expression responses to salt shock in maize roots. Plant Mol. Biol. 52, 873–891.

Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. Gen. Biol. 1 (6), reviews 3003.1–3003.9.

Winkel-Shirley, B., 2002. Biosynthesis of flavonoids and effects of stress. Curr. Opin. Plant Biol. 5, 218–223.