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Differential expression of genes coding for ABC transporters after treatment of *Arabidopsis thaliana* with xenobiotics

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Abstract ATP-binding cassette (ABC) transporters are thought to be involved in many cellular detoxification mechanisms. Performing a BLAST search, we found four distinct expressed sequence tags (EST) of *Arabidopsis thaliana* highly similar to the human and fungal glutathione-conjugate ABC transporters. We studied the expression of the corresponding genes in response to various xenobiotics in an effort to gain information on their function. The abundance of transcripts corresponding to one of the genes (*EST1*) was not affected by the various compounds tested, whereas the abundance of transcripts corresponding to the other three genes (*EST2, EST3, EST4*) was increased by 1-chloro-2,4-dinitrobenzene, primisulfuron and IRL 1803. Treatment of *Arabidopsis* with either primisufuron or IRL 1803 resulted in a more than 40-fold increase in *EST2*-specific transcripts.

Key words: ABC transporter; Expression; Xenobiotic; *Arabidopsis thaliana*

1. Introduction

Detoxification of xenobiotics and heavy metals, as well as of bacterial and fungal toxins, is of vital importance for the survival of plants exposed to such compounds. Well-known steps in this process are modifications of these substances by cytochrome P450-dependent monooxygenases and/or the conjugation to hydrophilic compounds such as glucose or glutathione [1]. For an efficient detoxification, excretion of modified toxic compounds, either into the apoplastic space or into the central vacuole, is required [2]. Excretion of glutathione conjugates into the vacuole has been shown to be mediated by a transporter which is directly driven by MgATP and is inhibited by vanadate [3,4].The vacuolar glutathione conjugate transporter activity increases in response to treatment of barley with safeners (herbicide antidotes) [5], a group of structurally diverse chemicals known to increase the overall tolerance of monocot plants towards herbicides [6], which indicates that transport of conjugated xenobiotics into the vacuole is an essential step in the cellular detoxification.

The known plant vacuolar glutathione conjugate transporter has properties very similar to those from animals and fungi [7,8]. However, in animal cells this transporter is located in the plasma membrane thus mediating excretion of the conjugates into the surrounding medium [7]. In human lung cancer cells, the multidrug-resistance-associated protein (MRP), a member of the ATP-binding-cassette (ABC) transporters has been shown to be a glutathione conjugate transporter [9]. In yeast, the ABC transporter YCF1 confers cadmium resistance. This protein is 42.6% identical to MRP. Yeast cells deficient for YCF1 exhibit high sensitivity to cadmium [10] and a strong decrease in glutathione-conjugate transport activity [11,12]. Recently it has been shown, that YCF1 also mediates the transport of the *bis*(glutathionato)cadmium complex [13]. Expression of MRP in YCF1-deficient yeast cells restores the cadmium tolerance and leads to an increased transport activity for glutathione-conjugates [12]. In contrast to YCF1, HMT1, another member of the ABC transporter family conferring cadmium tolerance in *Schizosaccharomyces pombe*, is not responsible for the transport of glutathione conjugates [14,15].

In yeast as well as in animals, many genes coding for ABC transporters have been identified [16], and a similar number of such genes can be assumed to exist in plants. The common motifs of all these transporters are the Walker A and B motifs [17] and the ABC domain [18]. The ABC transporters can structurally be subdivided into distinct classes, the one containing the MRP and YCF1 and the other the multi-drug-resistance proteins (MDR; also called P-glycoproteins) [19]. In an effort to identify genes encoding MRPs in plants and, furthermore, to possibly assign a physiological function to their products, we searched the dbest library of *Arabidopsis thaliana* and found four expressed sequence tags (EST) the deduced amino acid sequences of which are highly similar to those of MRP and YCF1. The corresponding genes are differentially expressed in response to treatment of *Arabidopsis* with several herbicides, heavy metals and other toxic compounds, indicating the involvement of different ABC transporters in different detoxification mechanisms.

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Abbreviations: IRL 1803, 3-hydroxy-3-(2*H*-(1,2,4)triazole-3-yl)-cyclohexyl-phosphonic acid; CDNB, 1-chloro-2,4-dinitrobenzene; PIPES, 1,4-piperazinediethanesulfonic acid; EST, expressed sequence tags; MRP, multidrug-resistance-associated protein; ABC, ATP-binding cassette

2. Materials and methods

2.1. Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB) and $CdCl_2$ were purchased from Fluka (Buchs, Switzerland). Primisulfuron, IRL 1803 (3-hydroxy-3-(2*H*-(1,2,4)triazole-3-yl)-cyclohexyl-phosphonic acid) and metolachlor were kind gifts from CIBA-GEIGY (Basel, Switzerland).

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2.2. Plant material

Arabidopsis thaliana (ecotype Columbia) was cultivated as described [20].

Seeds were germinated on MS medium (Murashige-Skoog, Duchefa, Haarlem, The Netherlands) containing 1.2% Agar (Bacto-agar Difco, Basel, Switzerland). One-week-old seedlings were transferred to 250 ml Erlenmeyer flasks containing 50 ml of liquid MS medium. Flasks were agitated at 100 rpm at a light/dark cycle of 16 h (21°C)/8 h (16°C). After 15 days compounds were added at final concentrations of: 100 μM CDNB, 10 μM $CdCl_2$, 20 nM primisulfuron, 5 μM metolachlor, 130 μM IRL 1803. Samples were harvested at 0, 12, 36 and 60 h after beginning of the treatment, frozen in liquid nitrogen and stored at −80°C until further use.

2.3. Southern blot analysis

Genomic *Arabidopsis thaliana* DNA was digested with the restriction enzymes *Eco*RI, *Hin*dIII or *Not*I and subjected to Southern blot analysis [20]. Transfer to Hybond N membranes (Amersham, Zürich, Switzerland) was performed according to the manufacturer's instructions. Prehybridization and hybridization were performed at 42°C in 50% formamide, 5×SSC (1×SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0); 20 mM PIPES, pH 6.4; 200 $\mu g \cdot ml^{-1}$ denatured carrier DNA; 2×Denhardt's solution (1×Denhardt's = 0.02% each of Ficoll 400, bovine serum albumin and polyvinylpyrrolidone), 0.5% SDS. The filters were washed twice for a few minutes in 0.3×SSC, 0.5% SDS at room temperature and 3 times for 30 min in the same buffer at 60°C. Autoradiography was performed with a PhosphorImager (Molecular Dynamics, Paul Bucher, Basel, Switzerland).

2.4. Radiolabeled probes

EST fragments were radioactively labeled according to the method of [22].

2.5. Northern blot analysis

RNA was isolated from frozen tissue according to [23]. Poly(A)$^+$ RNA was purified by passage over oligo(dT) cellulose (Qiagen, Basel, Switzerland). Denatured poly(A)$^+$ RNA (5 μg) was fractionated on a 1.2% formaldehyde agarose gel [21] and transferred to Hybond N membranes according to the manufacturer's instructions. Prehybridization and hybridization as well as autoradiography of the signals were performed as described for Southern blot analysis.

2.6. Dot-blot analysis

Dot blots were made using a Bio-Dot apparatus (Bio-Rad, Glattbrugg, Switzerland) as described in [24]. Four micrograms of total RNA, or corresponding amounts of calf liver RNA type IV (Sigma, Deisenhofen, Germany) were denatured in 6×SSPE (20×SSPE = 20 mM EDTA, pH 7.4, 3 M NaCl, 0.2 M sodium phosphate, pH 7.4) containing 20% deionized formaldehyde for 15 min at 55°C and then chilled on ice. Two volumes of ice cold 15×SSPE were added and the samples were applied to a GeneScreen membrane (NEN, DuPont, Bad Nauheim, Germany). Prehybridization and hybridization were performed as described for Southern blot analysis. The membranes were washed twice for a few minutes in 0.3×SSC, 0.5% SDS at room temperature and twice for 30 min in the same buffer at 60°C. Autoradiography and quantification of the signals were performed with a PhosphorImager. Fold induction was calculated by dividing the signal strength of the experimental sample by the signal strength of the 0 h value. All experiments were performed in duplicate, but data for only a single series are shown.

2.7. Isolation of potential full-length clones corresponding to EST2

To isolate potential full-length cDNA clones corresponding to EST2, a size selected *Arabidopsis thaliana* cDNA library in the vector λ ZAP (Stratagene, Zürich, Switzerland) was made and screened in duplicate using a ^{32}P-labelled EST2 fragment as probe. Twenty clones were plaque-purified and both strands of the largest cDNA clone (named AtMRP) were sequenced using the primer walking method [21].

Sequencing was performed using the dye terminator sequencing kit (Perkin Elmer, Küsnacht, Switzerland) and an ABI Prism 373A Sequencer (Perkin Elmer).

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          1                                                  50
EST2  ...VTYGLSL NTLQAWLIWT LCNLENKIIS VERILQYASV PSEPPLVIES
EST3
EST4  ...LSYALNI TSLLTGVLRL ASLAENSLNA VERVGNYIEI PPEAPPVIEN
YCF1  ...LSYALQI TQTLNWIVRM TVEVETNIVS VERIKEYADL KSEAPLIVEG
MRP   ...VSYSLQV TTYLNWLVRM SSEMETNIVA VERLKEYSET EKEAPWQIQE
CONS  ...VSY-L-- TT-LNW-VRM -S--E-N-V- VERVK-YA-- -SEAP-VIE-

          51                                                 100
EST2  NRPEQSWPSR GEVEIRDLQV RYAPHMPLVL RGITCTFKGG LRTGIVGRTG
EST3         ... GNVHLEDLKV RYRPNTPLVL KGITLDIKGG EKVGVVGRTG
EST4  NRPPPGWPSS GSIKFEDVVL RYRPQLPPVL HGVSFFIHPT DKVGIVGRTG
YCF1  HRPPKEWPSQ GDIKFNNYST RYRPELDLVL KHINIHIKPN EKVGIVGRTG
MRP   TRPPSSWPQV GRVEFRNYCL RYREDLDFVL RHINVTINGG EKVGIVGRTG
CONS  NRP--SWPS- G-VKFRDY-V RYRP--D-VL -GV--TIKGG EKVGVVGRTG
                                                           A

          101                                                150
EST2  SGKSTLIQTL FRIVEPSAGE IRIDGVNILT IGLHDLRLRL SIIPQDPTMF
EST3  SGKSTLIQVL FRLVEPSGGK IIIDGIDIST LGLHDLRSRF GIIPQEPVLF
EST4  AGKSSLLNAL FRIVEVEKGR ILIDDCDVGK FGLMDLRKVL GIIPQSPVLF
YCF1  AGKSSLTLAL FRMIEASEGN IVIDNIAINE IGLYDLRHKL SIIPQDSQVF
MRP   AGKSSLTLGL FRINESAEGE IIIDGINIAK IGLHDLRFKI TIIPQDPVLF
CONS  AGKSSLT-AL FRMVE-S-G- I-IDG--I-- -GLHDLR--- -IIPQDSV-F
       A

          151                                                200
EST2  EGTMRSNLDP LEEYTD.DQI WEALDKCQLG DEVRKKEQKL DSSVSENGDN
EST3  EGTVRSNIDP TEQYSD.EEI WKSLERCQLK DVVATKPEKL DSLVVDNGEN
EST4  SGTVRFNLDP FGEHND.ADL WESLERAHLK DTIRRNPLGL DAEVSEAGEN
YCF1  EGTVRENIDP INQYTDEAIW RALELSHLKE HVLSMSNDGL DAQLTEGGGN
MRP   SGSLRMNLDP FSQYSDEEVW T.SLELAHLK DFVSALPDKL DHECAEGGEN
CONS  SGTVRSNLDP --QY-D-A-W W-SL-R-HLK DVV--K-DKL D-SVSE-GEN

          201                                                250
EST1                ...RRSK ILVLDEATAA VDVRTDVLIQ KTIREEFKSC
EST2  WSMGQRQLVC LGRVLLKRSK ILVLDEATAS VDTATDNLIQ KTLREHFSDC
EST3  WSVGQRQLLC LGRVMLKRSR LLFLDEATAS VDSQTDAVIQ KIIREDFASC
EST4  FSVGQRQLLS LSRALLRRSK ILVLDEATAA VDVRTDALIQ KTIREEFKSC
YCF1  LSVGQRQLLC LARAMLVPSK ILVLDEATAA VDVETDKVVQ ETIRTAFKDR
MRP   LSVGQRQLVC LARALLRKTK ILVLDEATAA VDLETDDLIQ STIRTQFEDC
CONS  WSVGQRQLVC LGRA-LRRSK ILVLDEATAA VDVRTDA-IQ KTIRT-FK-C
            ABC                B

          251                                              296
EST1  TMLIIAHRLN TIIDCDKVLV LDSGKVQEFS SPENLLSNGE SSF...
EST2  TVITIAHRIS SVIDSDMVLL LSNGIIEEYD TPVRLLEDKS SSF...
EST3  TIISIAHRIP TVMDGDRVLV IDAGKAKEFD SPARLLERPS LFA...
EST4  TMLIIAHRLN TIIDCDKILV LDSGRVQEFS SPGEPSFK*
YCF1  TILTIAHRLN TIMDSDRIIV LDNGKVAEFD SPGQLLSDNK SLF...
MRP   TVLTIAHRLN TIMDYTRVIV LDKGEIQEYG APSDLLQQRG LFY...
CONS  T-LTIAHRLN TVMD-DRVLV LD-GKV-EYD SPGRLLSD-S SS-...
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Fig. 1. Alignment of the deduced amino acid sequences of four ESTs of *Arabidopsis thaliana* which show similarity to MRP and YCF1, two well-characterized ABC transporters of the MRP class from human and yeast, respectively. The two Walker A and B motifs and the ABC domains are highlighted as A, B and ABC, respectively. The Genbank accession numbers for the sequences are U96398, U92650, U96399, U96400 for EST1 to EST4 respectively.

3. Results and discussion

For a better understanding of the function of ABC transporters in the cellular detoxification of plants, we performed a BLAST search [25] in the *Arabidopsis thaliana* dbest library [26] and checked for sequences similar to the human (MRP) and yeast (YCF1) glutathione conjugate transporters. We found four distinct sequences highly similar to those of known glutathione conjugate transporters. The genes corresponding to these ESTs were named *EST1* (originally EST 39B12T), *EST2* (EST 107J19T7), *EST3* (EST 147I22T7) and *EST4* (EST ATTS1601), respectively (Fig. 1). The presented results will illustrate the high practical value of ESTs as probes for the analysis of gene expression.

Since the available sequences of these four ESTs were not all overlapping, we sequenced them completely to ensure that they correspond to four distinct genes. These sequences were indeed different, and the identities of the coding regions of these cDNAs with respect to MRP and YCF1 were in the range of 50% (Fig. 1). The sequence comparison indicated that the four plant sequences are more closely related to

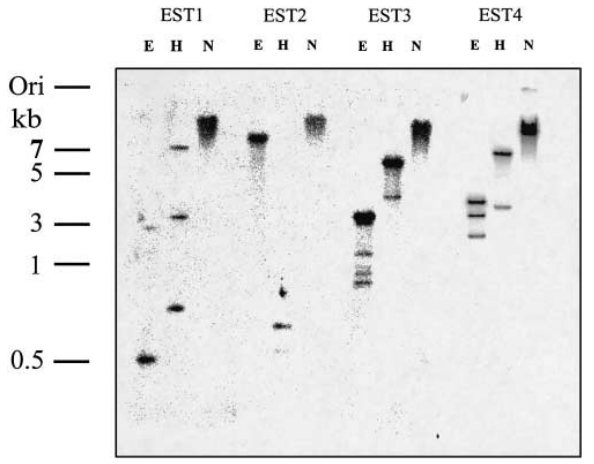

Fig. 2. Southern blot analysis with chromosomal *Arabidopsis* DNA. High molecular weight DNA was digested with the restriction enzymes *Eco*RI (E), *Hin*dIII (H) or *Not*I (N) and subjected to Southern blot analysis using the cDNAs corresponding to the ESTs as radiolabeled probes. The 1 kb ladder (BRL, Basel, Switzerland) was used as size marker. Ori: origin.

each other than to either MRP or YCF1. Genomic Southern blot analysis with *Arabidopsis* DNA indicated that all EST clones truly derived from *Arabidopsis* and that the corresponding sequences in the library are therefore not due to contaminating bacterial or fungal sequences in the cDNA library used to generate the dbest sequences (Fig. 2).

ABC transporters have been reported to occur both as 'half size' transporters (with molecular weights of 70–90 kDa) composed of one hydrophobic and one hydrophilic domain containing the ATP-binding cassette, and full size ABC transporters (140–170 kDa) which contain two domains each [16]. Northern blot analysis using the four cDNAs as probes revealed that the corresponding mRNAs have a uniform size of $\approx$5.5 kb, indicating that the corresponding genes code for full size ABC transporters (Fig. 3).

The expression of the different *ABC* transporter genes was

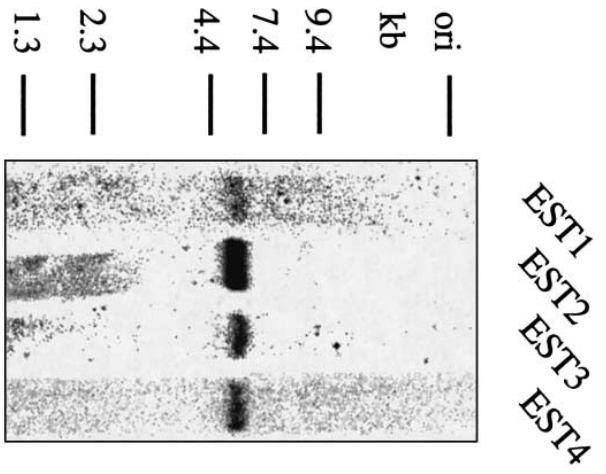

Fig. 3. Northern blot analysis of transcripts corresponding to the four ESTs. Poly(A)$^+$ RNA (5 μg) from *Arabidopsis* plants was subjected to Northern blot analysis using the same probes as for the Southern blot analysis (Fig. 2). RNAs of different length (BRL, Basel, Switzerland) were used as size markers (nucleotides $\times 10^{-3}$). Ori: origin.

studied in response to metolachlor, CDNB, primisulfuron, IRL 1803, and cadmium (Fig. 4) to gain information on their functions. The first step in the detoxification of metolachlor, an acetanilide-type herbicide, and of CDNB is their conjugation to glutathione. It had already been shown that treatment of *Arabidopsis* plants with CDNB resulted in a 2-fold increase in the activity of the vacuolar glutathione conjugate transporter [27]. Primisulfuron, a sulfonylurea-type herbicide which inhibits the biosynthesis of branched chain amino acids [28], is predominantly detoxified by conjugation to glucose after a hydroxylation reaction involving a cytochrome P450-dependent monooxygenase [29], while a minor part is hydrolyzed. IRL 1803, an experimental herbicide inhibiting histidine synthesis [20], is known to be only poorly inactivated in plants (K. Kreuz, personal communication), but a conjugation of IRL 1803 via the OH-group with glucose could potentially lead to its detoxification. Cadmium induces the synthesis of phytochelatins ($[\gamma\text{-glu-cys}]_n$-gly) which are involved in heavy-metal detoxification [30], and the synthesis of glutathione, the precursor of phytochelatins, is also enhanced in response to heavy metal exposure.

The strongest increase in the abundance of transcripts was observed for *EST2*-specific transcripts in plants treated with either primisulfuron or IRL 1803. Interestingly, these two compounds with completely different structures (Fig. 5), both of which may, however, be detoxified by glucosylation, induce the accumulation of the *EST2*-specific transcript. Therefore, it would be interesting to see whether both compounds also induce genes encoding other enzymes involved in detoxification processes (e.g. GST, cytochrome P450-depend-

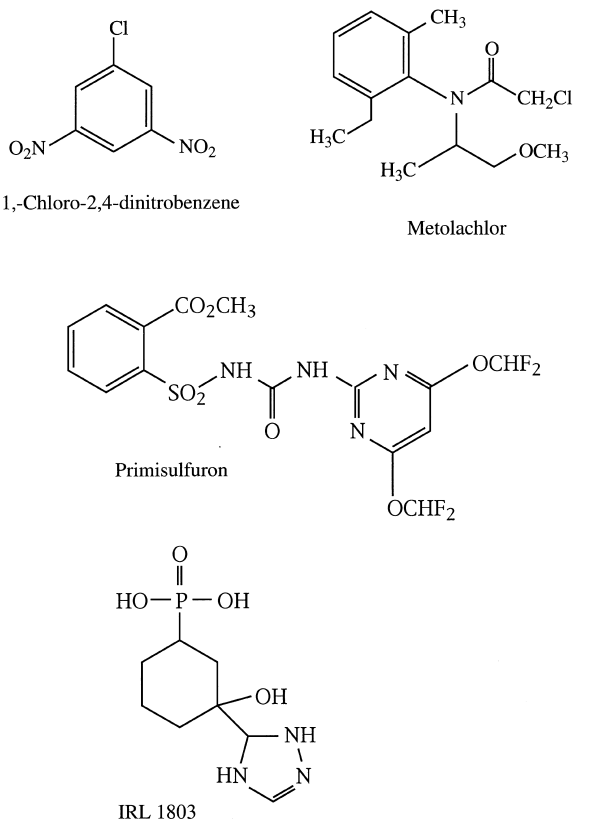

Fig. 4. Chemical structures of the xenobiotics with which *Arabidopsis* plants were treated to analyze the expression of the genes corresponding to the four ESTs.

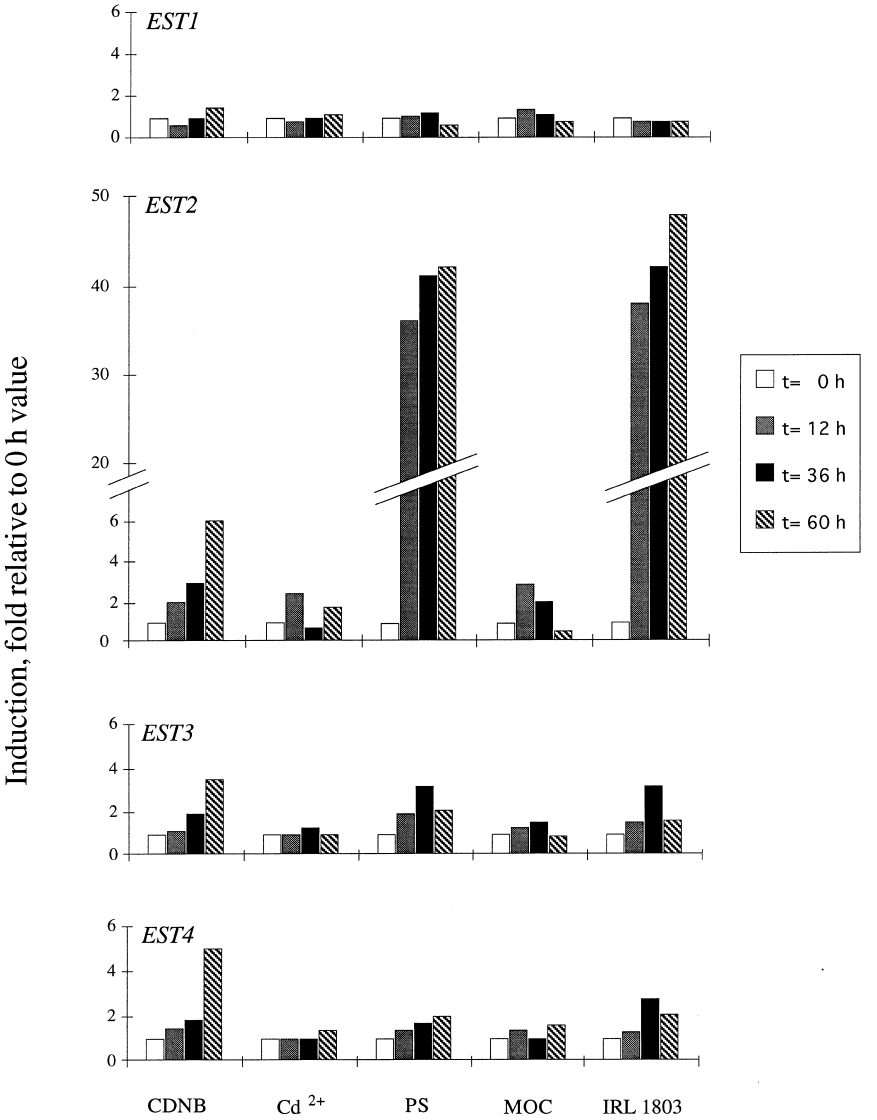

Fig. 5. Time courses of the relative abundances of *EST1*-, *EST2*-, *EST3*- and *EST4*-specific transcripts in *Arabidopsis* plants treated with either 1-chloro-2,4-dinitrobenzene (CDNB), cadmium (Cd^{2+}), primisulfuron (PS), metolachlor (MOC), or IRL 1803. Total RNA (5 μg) from plants inoculated with these xenobiotics for different periods of time was subjected to dot-blot analysis using the same probes as for Southern blot analysis (Fig. 2).

ent monooxygenase). IRL 1803 has been shown to induce the expression not only of genes of histidine biosynthesis but also of those involved in the synthesis of aromatic amino acids, lysine and purines [20], indicating the occurrence of cross-pathway regulation in plants. In the same study [20], an increase in transcripts corresponding to two enzymes involved in tryptophan biosynthesis, i.e. anthranilate synthase and phosphoribosyl-5-aminoimidazol synthetase, but not of enzymes involved in histidine biosynthesis, was also observed in response to primisulfuron treatment. Taken together with these results, our data indicate that the *EST2* gene product is either directly involved in the detoxification of primisulfuron and IRL 1803, or that it is involved in the transport of compounds (e.g. amino acid conjugates) which are only indirectly linked to the detoxification of the xenobiotics.

A 3- to 4-fold increase in abundance of *EST2*-specific transcripts was induced by CDNB. Cadmium and metolachlor had no significant effect, and this despite the fact that CDNB and metolachlor are detoxified via glutathione conjugation. Cadmium in contrast is detoxified by complexation with glutathione derivatives.

Comparably high increases in the abundance of transcripts were only observed for *EST3*- and *EST4*-specific transcripts of plants treated with CDNB. Under the conditions applied, the abundance of *EST1*-specific transcripts was not altered by any of the five xenobiotics tested.

The distinct accumulation pattern of certain *EST*-specific transcripts under defined conditions indicates that the expression of the corresponding genes is differentially regulated. Our results suggest that this regulation is not strictly correlated with the mode of detoxification of the respective xenobiotics. On one hand, primisulfuron, and probably also IRL 1803, which are detoxified as conjugates with glucose, strongly induce *EST2*, on the other hand CDNB, which is detoxified as

glutathione conjugate also induces *EST2, EST3* and *EST4* are induced not only by CDNB, but to a certain extent also by primisulfuron and IRL 1803 (Fig. 5).

These observations indicate that plants respond to toxic compounds, on the one hand, with an induction of proteins specifically involved in their detoxification, and on the other hand with an induction of proteins not obviously linked to such a function.

A putative full-length cDNA clone for EST2 (named AtMRP, accession number U92650) has been isolated and sequenced. At the amino acid level, AtMRP is 34% identical to both MRP and YCF1. Sequence comparisons (not shown) also revealed the second ATP binding domain common for proteins of the ABC family.

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