

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO			
RAD: 13-67186- -4	DEP: 2020 DIRECCION DE NUEVAS CREACIONES	FECHA: 2014-02-20 17:55:32	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	ACT: 519 PRESENTACION	FOLIOS: 8	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
LUZ CLEMENCIA DE PAEZ

Asunto: Radicación: 13-67186- -4
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 2164
 Folios: 8

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/EP2011/004383				
Fecha de Presentación Internacional	31/08/2011				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado 13-067186-00000-0000	3/Abril/2013
Reivindicaciones:	Radicado 13-067186-00000-0000	3/Abril/2013
Dibujos:	Radicado 13-067186-00000-0000	3/Abril/2013
Prioridad:	EP10009180.0	3/Septiembre/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por la solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10)

reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 15 del capítulo reivindicatorio (Radicado N°. 13-067186-00000-0000).

Título de la solicitud: “Reducción de Metal Pesado en Vegetales”.

El problema planteado en esta solicitud consiste en la necesidad de reducir el contenido de metales pesados, tales como cadmio, de vegetales o productos vegetales consumidos por animales y humanos.

Para resolver este problema técnico la solicitud en estudio proporciona polinucleótidos y polipéptidos que codifican transportadores ABC que se involucran en el transporte de metal pesado, modulación de la expresión o la actividad de las mismas.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 014/041

4. Excepción a la Patentabilidad (Art 20 D 486 literal (c))

El objeto de las reivindicaciones 1 – 4 y 6 no es patentable de acuerdo con el Art citado, ya que hace referencia a plantas, células vegetales, material vegetal, biomasa, semillas u hojas de la planta transformadas, se entiende que dichas semillas y partes de la planta hacen parte de una planta completa, por lo cual el alcance de estas reivindicaciones es una excepción a la patentabilidad.

5. De la solicitud de patente

Título

Se sugiere al solicitante modificar el título de la solicitud ya que el actual es muy general y no permite identificar la materia que contiene la solicitud.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 5 no es clara porque se refiere a un método para reducir niveles de cadmio, que es un resultado a alcanzar, puesto que la reivindicación incluiría no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado y además, menciona un procedimiento pero este no se define mediante características como alguna etapa y alguna condición específica en que debiera realizarse. Se sugiere reemplazarlo por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese efecto.

Las reivindicaciones 7, 10, 11, 13 y 14 definen el polipéptido, polinucleótido, constructo de polinucleótido y ARN bicatenario a partir de un porcentaje de homología lo que no permite establecer la secuencias nucleótidas exactas a la que se refieren y además, no hay sustento que demuestre que todas las posibles variables tengan la actividad que se quiere demostrar. Se sugiere definirlo a partir de la secuencias nucleótidas exactas indicando el número de la mismas (SEQ ID NO).

Las reivindicaciones 7 y 10 no son claras porque se refieren a que el polipéptido y polinucleótido tienen actividad transportadora de metal pesado, que es un resultado a alcanzar. Se sugiere definirlo correctamente.

El anticuerpo reclamado en la reivindicación 8 debe ser definido a partir de su estructura completa, esto es, de la secuencia nucleótida indicando el número de la misma (SEQ ID NO) o las secuencias de las regiones variables de la cadena ligera y cadena pesada y tener pleno sustento en la descripción.

El método de la reivindicación 9 no es claro porque no proporciona la metodología para

“ensayar” la presencia del polinucleótido de interés, el solicitante debe proporcionar la serie de pasos secuenciales lógicos que permitan determinar la presencia de un ácido nucleico, incluyendo reactivos, definiendo la secuencia del polinucleótido, cebadores y sondas a los que hace referencia y la descripción de los resultados positivos o negativos.

El vector reclamado en la reivindicación 17 debe ser descrito especificando por lo menos el tipo de vector y la secuencia recombinante que contiene

Se sugiere revisar y eliminar del capítulo reivindicatorio el término: “al menos”, debido a que es impreciso, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto.

Descripción (Art 28 D 486)

La descripción no divulga la invención de manera suficientemente completa y razonable que permita conocer y realizar la búsqueda de las secuencias reclamadas, también se desconocen las modificaciones realizadas a los aminoácidos y nucleótidos, tampoco hay sustento para las secuencias de las variables de los polipéptidos y polinucleótidos específicas probadas. En estos términos la descripción no es clara porque la información que contiene no permite que la persona del oficio normalmente versada en la materia pueda reproducirla o ejecutarla.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: septiembre 3 de 2010, se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO2009074325	“Transgenic Plants Modified for reduced Cadmium Transport, Derivative Products, And Related Methods”	18/Junio/2009
D2	Sanchez-Fernandez et al	“Cloning and Expression Analyses of ATMRP4, a Novel Mrp-Like Gene from Arabidopsis Thaliana”	01/Marzo/1998
D3	Genbank: FH224591	“CHO_OF3392xm19f1.ab1 CHO_OF3 Nicotiana tabacum genomic 5-, genomic survey sequence”	26/Octubre/2009
D4	WO03033705	“Transgenic Organism Expressing Fungal MRP-Like Abc Transporters”	24/Abril/2003

El documento D1¹ divulga plantas de tabaco transgénicas y semillas derivadas, modificados genéticamente para impedir el transporte de cadmio (Cd) del sistema de la raíz a las porciones aéreas de las plantas transgénicas mediante la reducción de los niveles de expresión de los transportadores relacionados con HMA (Resumen).

El documento D2² identifica proteínas MRP4 en Arabidopsis y familias cercanas tales como MRP3 (Resumen).

¹http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20090618&CC=WO&NR=2009074325A1&KC=A1

²Sanchez-Fernandez et al, 1998. ISSN: 0026-8925. Vol. 258, Pág. 655-662

El documento D3³ divulga secuencias de polinucleótidos de *Nicotiana tabacum* (Resumen).

El documento D4⁴ se refiere a ADN aislado que codifica proteínas MRP de transportadores ABC en organismos modificados confiriéndoles resistencia a y/o acumulación de materiales tóxicos tales como metales pesados, herbicidas, vectores y organismos transformados con el ADN aislado (Resumen).

Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 12 consiste en: *“Un ARN bicatenario que comprende al menos dos secuencias que son al menos parcialmente complementarias entre sí y en cuyo caso una hebra sentido comprende una primera secuencia y una hebra antisentido comprende una segunda secuencia y en cuyo caso al menos una de las secuencias comprende al menos 10 nucleótidos contiguos de NtMRP RNA.”.*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
ARN bicatenario	ARNi NtMRP (Pág. 59, Reivindicación 6)
Dos secuencias que son al menos parcialmente complementarias entre sí	Una primera secuencia que tiene al menos 70% de identidad de secuencia a una porción de SEQ ID NO: 3 o 47, una segunda secuencia, y una tercera secuencia (Pág. 59, Reivindicación 6)
Una hebra sentido comprende una primera secuencia y una hebra antisentido comprende una segunda secuencia	En el que la segunda secuencia está situada entre la primera secuencia y la tercera secuencia, y la segunda secuencia está operativamente ligado a la primera secuencia y a la tercera secuencia (Pág. 59, Reivindicación 6)
Una de las secuencias comprende al menos 10 nucleótidos contiguos de NtMRP RNA	La primera y la segunda secuencia tienen cada una una longitud seleccionada del grupo que consiste de 20-30 nucleótidos, nucleótidos 30-50, 50-100 nucleótidos, nucleótidos 100-150, 150-200 nucleótidos, 200-300 nucleótidos, nucleótidos 300 a 400, 400 a 500 nucleótidos, 500-600 nucleótidos, y 600 a 700 nucleótidos. (Pág. 61, Reivindicación 8)

Las características esenciales de la reivindicación 12 habían sido divulgadas previamente en el documento D1, el cual da conocer plantas transgénicas de tabaco modificadas genéticamente para expresar de forma estable polinucleótidos de RNAi que permiten la degradación de variantes endógenos de ARN de NtHMA.

En consecuencia, la reivindicación 12 no es nueva o carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 13 y 14 no mencionan alguna característica que haga nueva a la molécula de la reivindicación 12, por lo cual se considera que no son nuevas.

Nivel inventivo (Art18 D486)

La reivindicación 9 consiste en: *“Un método de detectar un polinucleótido NtMRP en*

3<http://www.ncbi.nlm.nih.gov/nucgss/FH224591>

4http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20030424&CC=WO&NR=03033705A1&KC=A1

una muestra que comprende el paso de:

(a) proporcionar una muestra que comprende un polinucleótido;

(b) poner en contacto dicha muestra con uno o varios cebadores o una o varias sondas para detectar específicamente al menos una porción de polinucleótido NtMRP; y

(c) detectar la presencia de un producto de amplificación, en cuyo caso la presencia de un producto de amplificación es indicativo de la presencia del polinucleótido NtMRP en la muestra”.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2
Un método de detectar un polinucleótido NtMRP en una muestra	Clonación y caracterización de AtMRP4 (Pág. 656, Col. 2)
Proporcionar una muestra que comprende un polinucleótido	Reacciones de PCR usando ADNc de Arabidopsis como molde (Pág. 656 Col. 2)
Poner en contacto dicha muestra con uno o varios cebadores o una o varias sondas para detectar específicamente al menos una porción de polinucleótido NtMRP	Cebadores ForI y RevI del ADNc de MRP humana (Pág. 656, Col. 2 a Pág. 657 Col. 1)
Detectar la presencia de un producto de amplificación, en cuyo caso la presencia de un producto de amplificación es indicativo de la presencia del polinucleótido NtMRP en la muestra”.	Produce un fragmento de PCR (Pág. 657 Col. 1)

El documento D2 se considera el estado de la técnica cercano porque divulga un método de clonación y caracterización de AtMRP4 que comprende reacciones de PCR usando ADNc de Arabidopsis como molde y los cebadores ForI y RevI basados en regiones altamente conservadas del ADNc de MRP humana que produce un fragmento de amplificación.

El método reivindicado difiere del documento D2 en el polinucleótido NtMRP detectado.

No existe evidencia de un efecto técnico inesperado al detectar el polinucleótido NtMRP puesto que el método comprende las mismas etapas.

Por lo tanto, el problema técnico objetivo a resolver consiste en: cómo obtener un método mejorado para la detección de un polinucleótido NtMRP en una muestra.

Sin embargo, la aplicación de PCR para la detección de un gen determinado en una planta corresponde a la aplicación de medios técnicos conocidos y rutinarios. De manera que el detectar un gen distinto en una planta mediante técnicas propias del quehacer de la persona normalmente versada en la materia, tal como lo divulga el documento D2, resulta obvio.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el polinucleótido NtMRP en el procedimiento divulgado en el documento D2 para llegar así al objeto de la reivindicación 9 de la solicitud. Por lo cual se considera obvia.

Conclusión:

La reivindicación 9 cumple el requisito de novedad (Art. 16), y no con el requisito de nivel inventivo (Art. 18).

La reivindicación 10 consiste en: “Un polinucleótido aislado seleccionado del grupo constituido por:

un polinucleótido aislado que comprende, consiste o esencialmente consiste en una secuencia que tiene al menos 71 % de identidad de secuencia con SEQ ID NOs: 1, 2,

27,
28 o 29 o 51;
un polinucleótido aislado que comprende, consiste o esencialmente consiste en una secuencia que tiene al menos 65 % de identidad de secuencia con cualquiera de SEQ ID
NOs: 3 a 23 o 30 a 50 o 53;
un polinucleótido que codifica un polipéptido NtMRP que comprende, consiste o esencialmente consiste en una secuencia que tiene al menos 65% de identidad de secuencia con cualquiera de SEQ ID NOs. 24 a 30 26 o 52, y en cuyo caso el polipéptido tiene actividad transportadora de metal pesado”.

La reivindicación 7 consiste en: “Un polipéptido NtMRP aislado, expresado por un polinucleótido seleccionado del grupo constituido por:
(i) un polinucleótido que comprende, consiste o esencialmente consiste en una secuencia que tiene al menos 71 % de identidad de secuencia con SEQ ID NOs: 1, 2, 27, 28 o 29 o 51; o
(ii) un polinucleótido que comprende, consiste o esencialmente consiste en una secuencia que tiene al menos 65 % de identidad de secuencia con cualquiera de SEQ ID NOs: 3 a 23 o 30 a 50 o 53;
o
un polipéptido NtMRP aislado que comprende, consiste o esencialmente consiste en la secuencia descrita en cualquiera de SEQ ID Nos. 24 a 26 o 52, en cuyo caso el polipéptido tiene actividad transportadora de metal pesado”

La reivindicación 11 consiste en: “Un constructo de polinucleótido de al menos 15 nucleótidos contiguos de longitud que es al menos 65 % idéntica a una región de cualquiera de SEQ ID NOs: 1 a 23 o 27 a 51 o 53”.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D3	D4
Polinucleótido aislado	Polinucleótido	ADN MRP (Pág. 24)
Un polinucleótido que comprende, consiste o esencialmente consiste en una secuencia que tiene al menos 71 % de identidad de secuencia con SEQ ID NOs: 1, 2, 27, 28 o 29 o 51; (ii) un polinucleótido que comprende, consiste o esencialmente consiste en una secuencia que tiene al menos 65 % de identidad de secuencia con cualquiera de SEQ ID NOs: 3 a 23 o 30 a 50 o 53; un polipéptido NtMRP aislado que comprende, consiste o esencialmente consiste en la secuencia descrita en cualquiera de SEQ ID Nos. 24 a 26 o 52, en cuyo caso el polipéptido tiene actividad transportadora de metal pesado	Genbank: FH224591	Proteínas YCF1, YHL035C, BPT1, YBT1 y YOR1 (Pág. 24)

El documento D3 divulga secuencias polinucleótidas que comprenden una secuencia 100% idéntica a la SEQ ID NO: 16 de la presente invención, y además las variantes de las mismas presentan un 71% de homología (58% de identidad) a NtMRP4.

La diferencia entre la invención, definida en la reivindicación 1 y los polinucleótidos divulgados de D3 consiste en el polipéptido NtMRP.

El efecto que logra el polipéptido NtMRP es que reduce el nivel medio de cadmio a nivel subcelular en plantas.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo obtener variantes de polinucleótidos de MRP alternativas para reducir el contenido de metales pesados, tales como cadmio, de vegetales o productos vegetales consumidos por animales y humanos.

Sin embargo, la utilización del polipéptido NtMRP para reducir el contenido de cadmio de vegetales, ya está divulgada en el documento D4 que identifica variantes de tabaco para producir plantas transformadas con MRP para la reducción de la acumulación de cadmio.

En consecuencia, la persona del oficio normalmente versada en la materia, generaría múltiples variaciones de promotores recombinantes empleando técnicas convencionales y métodos de biología molecular, biología vegetal y bioinformática de acuerdo a las enseñanzas de los documentos D3 y D4 para llegar así al objeto de la reivindicación 7 de la solicitud. Por lo cual se considera obvia.

Conclusión:

Las reivindicaciones 10, 7 y 11 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

Puesto que las reivindicaciones dependientes 5, 8 y 15 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

7. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2009074325	12 – 14	Novedad
D2	Sanchez-Fernandez et al	9	Nivel inventivo
D3	Genbank: FH224591	5, 7, 8, 10, 11 y 15	Nivel inventivo
D4	WO03033705	5, 7, 8, 10, 11 y 15	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-02-25

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ORIGINAL PAPER

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Cloning and expression analyses of *AtMRP4*, a novel *MRP*-like gene from *Arabidopsis thaliana*

Received: 20 February 1998 / Accepted: 9 March 1998

Abstract In all organisms glutathione-conjugate transporters (GS-X pumps) mediate the detoxification of a number of xenobiotics by removing them from the cytosol. In addition, GS-X pumps appear to play a role in the processing of endogenous compounds. We have isolated a novel genomic clone from *Arabidopsis thaliana* that encodes a putative GS-X pump, *AtMRP4*, which is part of a recently defined gene family. The derived amino acid sequence shares high levels of similarity (55–63%) with human, yeast, and other *Arabidopsis* homologues. The expression of the different members of the *AtMRP* gene family in *Arabidopsis* cell suspensions after treatment with chemicals that modify glutathione metabolism (compounds that induce different types of stress and that act as herbicide antidotes – safeners – in monocotyledonous species) revealed that the members of this gene family are differentially regulated.

Key words *Arabidopsis thaliana* · Glutathione-conjugate pump · Stress · Xenobiotic detoxification

Introduction

In higher plants, interest in glutathione (γ -glutamylcysteinyl glycine, GSH) has been largely focused on its role in the removal of active oxygen species that may accumulate during environmental stresses, such as exposure to air pollutants, drought and extremes of temperature (Alscher 1989). Induction of GSH synthesis in response to stress appears to be an important component of plant defence strategies (May and Leaver 1992). As an adaptive response, the activation of such defences may contribute to the survival of plants in a changeable and often hostile environment by minimizing damage to cellular components. An additional line of defence is provided by mechanisms for the removal of xenobiotics and the products of cellular damage, such as lipid peroxides. GSH is involved in the elimination of the highly toxic derivatives of lipid peroxidation (Ishikawa et al. 1986) and in the sequestration of heavy metals (Alscher 1989).

Detoxification of xenobiotics in plants is accomplished by a process that resembles that seen in other organisms, and is divided in three phases: activation, conjugation, and transport of the xenobiotic. When detoxification occurs through conjugation to GSH, the transport of the glutathione-xenobiotic conjugate (GS-X) takes place via specific ATP-dependent transporters (GS-X pumps) that belong to the ATP-binding cassette (ABC) superfamily. In human cell cultures, the multidrug resistance-associated protein (MRP) is responsible for the detoxification of several anticancer drugs (Cole et al. 1992). MRP is also involved in the transport of conjugates of GSH with endogenous substrates, such as leukotrienes and sterols (Loe et al. 1996a, b). A similar transport system has been reported in the vacuolar membrane of the yeast *Saccharomyces cerevisiae* (Szczycka et al. 1994). This transporter, yeast cadmium factor 1 (YCF1), is involved in the detoxification of cadmium via sequestration of GS-cadmium conjugates (Li et al. 1996). The expression of both the human *MRP*

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and the yeast *YCF1* is highly regulated and coordinated with other aspects of GSH metabolism (Ishikawa et al. 1994; Zhu and Center 1996; Moradas-Ferreira et al. 1996); they may also fulfil similar functions, as a yeast *ycf1*⁻ mutant can be functionally complemented by the human *MRP* cDNA (Tommasini et al. 1996).

In plants, a GS-X pump involved in similar detoxification mechanisms has been biochemically characterized in the vacuolar membrane (Martinoia et al. 1993; Li et al. 1995). A function has also been attributed to these transporters during normal plant growth and development, because they are involved in cellular reactions that imply conjugation of GSH with endogenous substrates, such as anthocyanins (Marrs et al. 1995) and plant hormones (Li et al. 1997). Recently the genes encoding three *Arabidopsis* GS-X transporters have been cloned: *AtMRP1* (Lu et al. 1997), *AtMRP2* (Lu et al. 1998), and *AtMRP3* (Tommasini et al. 1997). *AtMRP1* acts as a low-affinity GS-X transporter in the vacuolar membrane and can complement a *ycf1*⁻ yeast mutant (Lu et al. 1997). Both *AtMRP1* and *AtMRP3* share high levels of similarity with GS-X transporters from other organisms. The existence of an *Arabidopsis* *MRP* gene family raises the question of the functional specialization of different members of the family.

To gain a more complete understanding of the function and regulation of the *AtMRP* gene family, a novel gene for a putative *A. thaliana* GS-X transporter, *AtMRP4*, was cloned and characterized. We also analyzed the effect of compounds that modify GSH metabolism on the expression of *AtMRP* genes. Two groups of compounds were chosen: stress inducers, and herbicide antidotes (safeners) used in monocotyledonous species. Our data reveal differential expression of *AtMRP* genes.

Materials and methods

Chemicals

Compounds that act as herbicide antidotes (safeners) in monocotyledonous species, such as benoxacor [(±)-4-dichloroacetyl-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine; Ciba-Geigy], cloquintocet [1-methylhexyl(5-chloro-quinolin-8-yloxy)acetate; Ciba-Geigy], fenchlorazole [1-(2,4-dichloro-phenyl-5-trichloromethyl-1*H*-1,2,4-triazole-3-carboxylic acid; AgrEvo], flurazole (benzyl 2-chloro-4-trifluoromethyl-1,3-thiazole-5-carboxylate; Monsanto) and oxabetrinil [(*Z*)-1,3-dioxolan-2-ylmethoxyimino(phenyl)acetonitrile; Ciba-Geigy] were kindly provided by David Cole (Rhône-Poulenc Agriculture Ltd, Essex, England). Aminotriazole (3-amino-1,2,4-triazole), menadione (vitamin K₃: 2-methyl-1,4-naphthoquinone) and cadmium sulphate were purchased from Sigma Chemicals (St. Louis, Mo.).

Cloning of *AtMRP4*

The degenerate primers *ForI* (5'-AAGGTCGGCA-TIGTIGGGCGGA-3') and *RevI* (5'-AGCTGICGCTGCCCCI-CICT-3'), corresponding to the highly conserved regions 4160-4506 of the human *MRP* cDNA (Cole et al. 1992) and 4115-4461 of the *Leishmania tarentolae* *lTPGPA* gene (Ouellette et al. 1990), were used at a final concentration of 0.3 pmol/μl in a PCR reaction with

15 ng/μl of an *Arabidopsis* cDNA library (Minet et al. 1992) as a template, as described previously (Mayer et al. 1995). A PCR fragment of the predicted size (347 bp) was obtained after 5 min at 94°C followed by 35 cycles at 94°C (1 min), 55°C (1 min) and 72°C (2 min), cloned in pGEMT (Promega, Madison, Wis.), sequenced and used to search the EST (expressed sequence tag) database using the BLAST algorithm [Genetics Computer Group (GCG), Madison, Wis.]. One EST clone, 147I22T7, showed 100% identity to the PCR fragment. This clone (1108 bp) was used as a probe to screen an *Arabidopsis* genomic library in λGEM11 (Schna and Davis, 1992). From a total of 200 000 phage plaques, 100 primary positives were detected under highly stringent conditions (Sambrook et al. 1989). Of these, 10 were purified and sequenced directly in the λ vector. One clone of 15 kb was identified in which the zone of homology with the probe was adjacent to the right arm of λGEM11. Thus, the presence of the full-length *AtMRP4* gene in this clone was assured. The recombinant phage was digested with *EcoRI* and *SacI*, and the fragments obtained subcloned in pBluescript (Stratagene, Madison, Wis.) and fully sequenced on both strands using fluorescent dye terminators and AmpliTaq FS polymerase (Perkin Elmer, Norwalk, Conn.) on an ABI373 A automatic DNA sequencer (Applied Biosystems, Foster City, Calif.).

Plant material and treatments

For expression analyses, *A. thaliana* (L.) Heynh. Col-0 cell cultures, kindly provided by Dr. M. Axelos (Toulouse, France), were used and maintained essentially as described (Glab et al. 1994). Exponentially growing cultures were used for all treatments. All compounds were used at concentrations previously shown to increase GSH synthesis: 10 μM for safeners (May et al., in preparation), 2 mM for aminotriazole (May and Leaver 1992), 20 μM for menadione (J.-P. Reichheld, personal communication), and 20 μM for cadmium sulphate (H. Schäfer, personal communication). In all experiments, 2-ml samples of cell suspensions were collected at the indicated times after the addition of the chemicals and used for RNA purification.

RNA purification and Northern analysis

Total RNA was extracted from 2-ml samples of *Arabidopsis* cell suspensions using TriZol reagent (Gibco BRL, Gaithersburg, Md.) according to the manufacturer's instructions. Denatured total RNA (15 μg) was fractionated on a 1.2% agarose gel and transferred to uncharged nylon membranes (Qiabrane; Qiagen, Chatsworth, Calif.). Before hybridization, the membranes were stained with methylene blue to confirm equal transfer of the RNA (Sambrook et al. 1989). Hybridizations with radiolabeled EST DNA under stringent conditions were performed according to standard procedures (Sambrook et al. 1989). Quantitation of probe hybridization was performed with a phosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

EST clones

EST fragments identical to the published *Arabidopsis* *MRP* genes were used as probes in expression analyses: ATTS1246, corresponding to *AtMRP1* (Lu et al. 1997), 107J19T7, corresponding to *AtMRP3* (Tommasini et al. 1997), and 147I22T7, corresponding to *AtMRP4* (this paper).

Results and discussion

Cloning and characterization of *AtMRP4*

PCR reactions using *Arabidopsis* cDNA as template and the primers *ForI* and *RevI* (Mayer et al. 1995), based on

highly conserved regions of the human *MRP* cDNA (Cole et al. 1992) and the *Leishmania tarentolae* *ltPGPA* gene (Ouellette et al. 1990), produced a fragment of the predicted size (347 bp). The PCR fragment was cloned in pGEMT, sequenced, and used to search the EST database using the BLAST program. An EST fragment of 1 kb (147I22T7) was identified with 100% identity to the 347-bp PCR fragment. The EST fragment was fully sequenced and proved to be, as expected, the 3' end of the gene.

The EST fragment 147I22T7 was used as probe in a Southern analysis of *Arabidopsis* (Col-0) genomic DNA digested with *Eco*RI, *Hind*III, or *Sal*I. We confirmed the previously published finding (Tommasini et al. 1997) that the gene was present as a single copy in the *Arabidopsis* genome and the probe did not cross-hybridize with other *Arabidopsis* *MRP* genes under conditions of high stringency (data not shown).

The EST fragment 147I22T7 was then used to screen a genomic library from *Arabidopsis* Col-0 in λ GEM11 (Schena and Davis 1992). Approximately 100 positives were obtained in a primary screen of 200 000 plaques, and DNA from 10 of these positives was sequenced directly in the λ vector. One clone, λ 6, was fully sequenced because it presented the whole EST sequence adjacent to the right arm of λ GEM11 in the correct orientation. The genomic fragment, of approximately 15 kb, contained the promoter and complete coding sequence of a novel putative ABC transporter that we named *AtMRP4*. It contained also another novel gene whose deduced product showed significant homology (51.12% similarity and 28.12% identity) with *Arabidopsis* *SCARECROW* (Di Laurenzio et al. 1996), and a truncated *Myb* gene (Fig. 1A). According to a coding sequence prediction executed with the program NetPlantGene (Hebsgaard et al. 1996), the *AtMRP4* genomic clone contained 11 exons and 10 introns (Fig. 1A). The putative start codon selected by the program matches the consensus sequence for *Arabidopsis* genes (S. Rombauts, personal communication).

The predicted polypeptide of 1516 amino acids encoded by *AtMRP4* (Fig. 1B) has an estimated molecular mass of 169 kDa and an estimated isoelectric point of 7.95. The structure of *AtMRP4* indicates that it belongs to the superfamily of ABC transporters, specifically to the MRP subgroup. *AtMRP4* presents a clear ABC transporter signature consisting on two nucleotide-binding folds (NBF1 and NBF2), each containing the Walker A and B motifs and C domain (Higgins 1992). In addition, two subclass-specific structures that classify the protein as a MRP were evident: a putative regulatory (R) domain rich in charged amino acids that follows NBF1, and an N-terminal extension of 246 amino acids (Fig. 1B). According to a computer prediction performed with the program SOSUI (Mitaku et al. submitted), *AtMRP4* has 17 transmembrane helices. The protein topology is consistent with a model proposed recently for the human MRP with three well-defined transmembrane blocks (Bakos et al. 1996; Hipfner et al.

1997). The first block, with five transmembrane helices, is located within the N-terminal extension (Hipfner et al. 1997). The other two blocks, each consisting of six transmembrane spans, flank the NBF1 and R domains and represent the two transmembrane domains characteristic of ABC transporters, TM1 and TM2 (Bakos et al. 1996; Hipfner et al. 1997) (Fig. 1B). In short, the predicted structure of *AtMRP4* is compatible with the theoretical structure predicted for other ABC transporters belonging to the MRP subclass: N-TM1-NBF1-R-TM2-NBF2-C, where C represents the C-terminal region of the protein.

Comparisons of *AtMRP4* with other ABC transporters revealed a very significant degree of overall identity (33–41%) and similarity (54–63%) with all proteins with GS-X transport activity described so far, and lower levels of identity (approximately 30%) and similarity (53–55%) with cystic fibrosis transmembrane conductance regulators (CFTRs) from different organisms and the *L. tarentolae* P-glycoprotein-related molecule (*ltPGPA*) (Table 1). This is rather surprising, in view of the fact that the gene encoding the latter was one of the two sequences used in the design of the degenerate PCR primers used in the first step of the cloning of *AtMRP4*. On comparing the putative domains of *AtMRP4* with their equivalents in other proteins, the highest similarity levels were found in both NBFs, particularly in NBF2, where the score reached 84%. The lowest identity and similarity levels were found in the putative regulatory domain R, with identities between 24% and 38% (Table 1). *AtMRP4* exhibited most similarity at all levels (overall and divided in domains) to *AtMRP3*, whereas the degree of identity and similarity shared with *AtMRP1* is comparable to that of GS-X pumps from other organisms (Table 1).

A dendrogram of representative members of the ABC transporters superfamily, derived from a Pileup analysis (GCG; default parameters), showed a division of the phylogeny tree in two branches, the first one consisting of the P-glycoproteins, major histocompatibility transporters, and the yeast pheromone transporter STE6; and the second one including the CFTRs, sulfonylurea receptor (SUR), the *Leishmania* PGPA, and all proteins with GS-X transport activity (MRPs, cMOATs, and YCF1). Figure 2 shows only the second branch of the ABC transporter phylogeny. It is evident that *AtMRP3* and *AtMRP4* appear to define a subgroup within the MRP subclass, based on their relatively early separation from all the other GS-X pumps known to date, including *AtMRP1*. This, together with the data shown in Table 1, could be an indication of functional differentiation within the *AtMRP* protein family.

Expression analyses

Sequence similarities to and differences from previously described clones (Lu et al. 1997, 1998; Tommasini et al. 1997) suggested specialization within the *Arabidopsis*

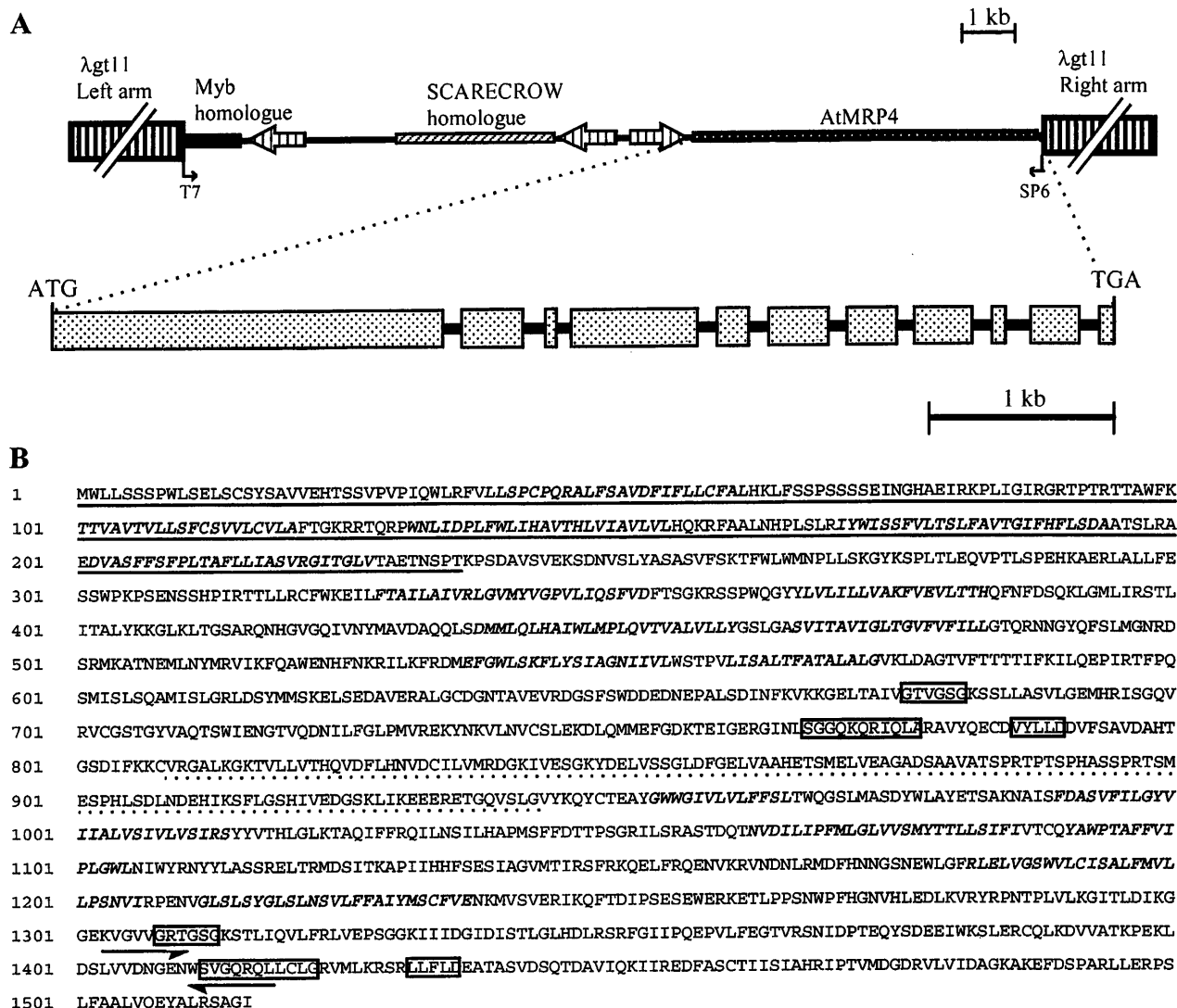


Fig. 1A Structure of the genomic clone $\lambda 6$ and organization of the *AtMRP4* gene. $\lambda 6$ contains the full-length *AtMRP4* gene and two other ORFs: a full-length homologue of *SCARECROW* and a truncated gene encoding a Myb domain. Promoters are represented by arrows. The relative positions of the predicted exons and introns of the *AtMRP4* genomic clone are indicated by stippled boxes and thick lines, respectively. **B** Deduced amino acid sequence of the polypeptide encoded by *AtMRP4*. The N-terminal extension (amino acids 1-246) is indicated by the underlined Roman typeface. Putative transmembrane helices, identified using the SOSUI program (Mitaku et al. submitted), are indicated in bold italics. The Walker A (sequences GTVGSG and GRTGSG) and B boxes (sequences VILLD and LLFLD), and the ABC signature motifs (SGGQKQRIQLA and SVGQRQLLCLG) are boxed. The CFTR-like putative regulatory domain (R; amino acids 809-941), is underlined with dots. The positions and directions of the degenerate PCR primers used to amplify *A. thaliana* cDNA during the initial steps of the cloning of *AtMRP4* are indicated by the arrows

MRP gene family. This is of interest in light of the findings described in other systems. In human cells several *MRP* homologues have been identified, and tissue-specific expression has been reported (Kool et al. 1997).

Also, there is co-regulation of *MRP* with other GSH-related genes, such as the γ -glutamylcysteine synthetase gene (γ -ECS) and genes encoding glutathione-S-transferase (GSTs; Ishikawa et al. 1994), and during responses to oxidative stress (Lenehan et al. 1995), heavy metals (Ishikawa et al. 1996) and heat shock (Kondo et al. 1993).

To investigate the putative specialization of the members of the *AtMRP* gene family, expression analyses were performed. *Arabidopsis* cell suspensions were used because they facilitate the simultaneous manipulation of many treatments and samples under controlled conditions, represent a relatively homogeneous system, and permit kinetic studies of responses to different treatments.

Arabidopsis cell suspensions were treated with stress agents at sublethal doses that have previously been shown to increase GSH synthesis (see Materials and methods). Aminotriazole leads to mild oxidative stress through inhibition of catalases, whereas menadione causes oxidative stress through generation of superoxide. Cadmium was used as an example of heavy metal

Table 1 Percentage identity and similarity of AtMRP4 to other ABC transporters

		Average ^a	N	TM1	NBF1	R	TM2	NBF2	C
AtMRP1	Identity	33.9	15.6	29.9	52.7	33.3	28.7	56.4	49.2
	Similarity	56.3	41.3	50.6	76.0	53.9	55.0	72.1	67.2
AtMRP3	Identity	41.1	18.8	35.9	65.3	38.1	38.5	66.1	50.0
	Similarity	62.9	47.3	59.9	81.3	57.9	60.3	84.2	63.3
ScYCF1	Identity	33.0	14.3	27.6	51.7	24.4	32.0	55.8	48.3
	Similarity	54.5	44.4	49.5	64.4	44.3	55.7	73.3	65.0
rtCMOAT	Identity	36.8	25.0	28.1	58.4	30.8	34.9	58.7	38.3
	Similarity	59.7	52.1	53.5	74.5	53.4	58.8	75.0	63.3
hmMRP	Identity	35.0	22.7	31.6	50.0	31.1	31.9	57.0	39.3
	Similarity	56.5	47.1	52.1	70.0	51.5	56.3	73.3	64.3
LtMRP	Identity	30.6	20.3	19.9	46.7	29.2	29.9	49.4	35.6
	Similarity	53.3	38.3	44.4	63.3	55.0	53.4	72.7	66.1
hmCFTR	Identity	30.6	0	24.7	43.3	20.0	27.7	41.2	35.0
	Similarity	55.6	0	51.7	62.0	40.8	53.6	67.7	58.3

^a Comparisons were done with Bestfit (GCG, default parameters)

stress. The compound fenchlorazole (Fig. 3), which acts as a herbicide antidote (safener) in monocotyledonous species, was used because of its potential activity as a modulator of GSH metabolism in *Arabidopsis*, because we have found that it acts in a GSH-dependent manner

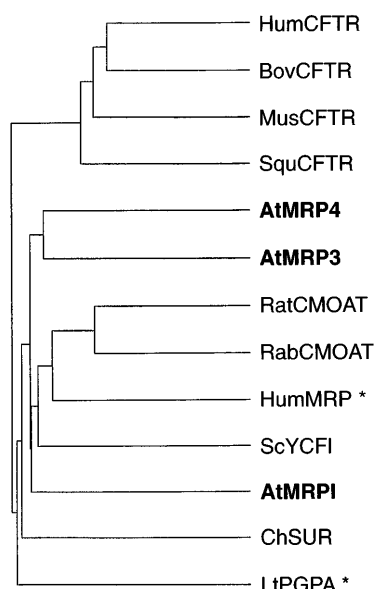


Fig. 2 Phylogenetic tree of the MRP-CFTR subclass of ABC transporters. All P-glycoproteins belong to an independent branch of the dendrogram (not shown). AtMRPs are in **bold**. The asterisks indicate the proteins utilized in the design of the degenerate PCR primers used in the initial steps of the cloning of *AtMRP4*. The EMBL/Genbank accession numbers of the sequences used in the construction of the dendrogram are: cystic fibrosis transmembrane conductance regulators from humans (HumCFTR, A30300), cow (BovCFTR, M76128), mouse (MusCFTR, M69298), and dogfish (SquCFTR, M83785); multidrug resistance-associated proteins from *Arabidopsis* (AtMRP1, AF008124, AtMRP3, U92650 and AtMRP4, AJ002584) and humans (HumMRP, L056028); canalicular multispecific organic anion transporters from rat (RatCMOAT, L49379) and rabbit (RabCMOAT, Z49144); *Saccharomyces cerevisiae* yeast cadmium factor (ScYCF1, L35237); Chinese hamster sulfonyleurea transporter (ChSUR, L40623) and *Leishmania tarentolae* P-glycoprotein-related protein (LtPGPA, X17154)

(May et al., in preparation). We wished to test whether co-regulation of *AtMRP4* with other members of the *AtMRP* family occurs under these conditions. *Arabidopsis* cell suspensions were incubated with the chemicals and 2-ml samples were collected for RNA purification 0, 1, 2, 4, and 6 h after the addition of the chemicals. After gel separation of 15 µg of denatured total RNA, steady-state mRNA levels corresponding to *AtMRP1*, *AtMRP3*, and *AtMRP4* were determined by Northern analysis using EST clones as probes (ATTS1246 for *AtMRP1*, 107J19T7 for *AtMRP3*, and 147I22T7 for *AtMRP4*) (Fig. 4). Both *AtMRP3* and *AtMRP4* were induced by oxidative stress, but showed a differential response depending on the source of the oxidant. Whereas the levels of *AtMRP3* mRNA increased in the presence of aminotriazole, *AtMRP4* responded to the treatment with menadione. Treatments

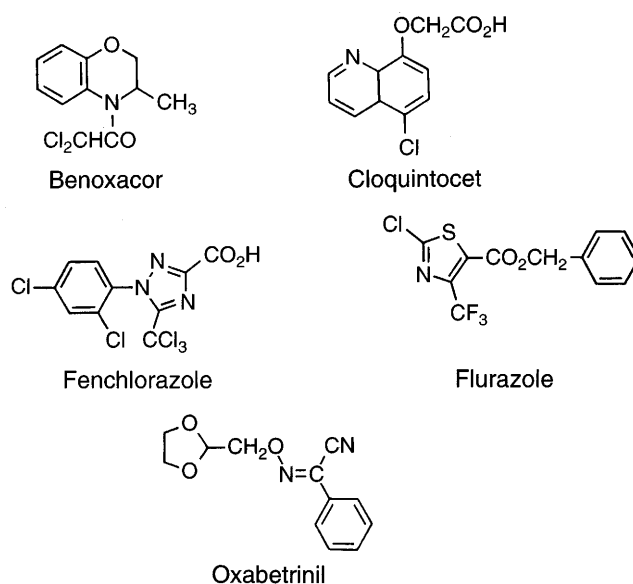


Fig. 3 Chemical structure of the herbicide antidotes (safeners) with which *Arabidopsis* cell suspensions were treated in order to analyze the expression of the *AtMRP* genes

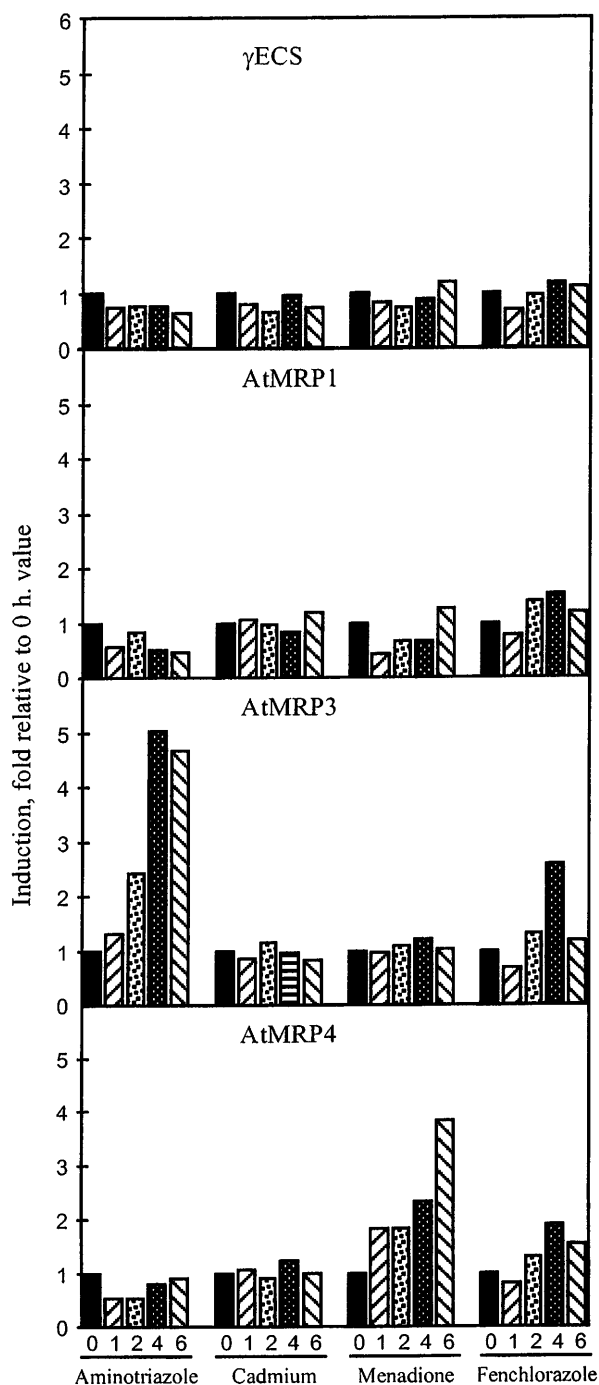


Fig. 4 Changes in the levels of *AtMRP1*, *AtMRP3*, and *AtMRP4* mRNAs after treatment of cell suspensions with different compounds for 1, 2, 4, or 6 h, as quantified by phosphor-imaging. The levels of expression of the γ -ECS gene, encoding the first enzyme in the biosynthesis of GSH, are shown as a constitutive control

with Cd did not affect the expression level of any of the genes tested. *AtMRP1* was not significantly induced under any of the conditions used. As a constitutive control, a probe corresponding to the *Arabidopsis* γ -ECS gene, whose expression does not change in response to any of these treatments (May et al., in preparation), was used (Fig. 4). Induction of members of the *AtMRP* gene

family by oxidative stress is consistent with findings in other organisms and suggests that, in *Arabidopsis*, some specific members of the MRP family may play a role in tolerance to oxidative stress. In the yeast *S. cerevisiae*, oxidative stress leads to increased expression of the transcription factor yAP-1, which is a transcriptional activator of γ -ECS and *YCF1* (Moradas-Ferreira et al. 1996). Similarly, human MRP is regulated by SP1 (Zhu and Center 1996), a transcription factor that also activates Pi class GSTs (Moffat et al. 1996).

More extensive expression analyses were performed using herbicide antidotes (safeners) (Fig. 3) known to stimulate different aspects of GSH metabolism in monocotyledonous species. Benoxacor, flurazone, and oxabectrinil increase the activity of GSTs in maize (Wiegand et al. 1986; Irzyk and Fuerst 1993) and cloquintocet increases the activity of a GS-X pump in barley by almost twofold (Gaillard et al. 1994). Although there is no evidence to date that these compounds act as "safeners" in dicotyledonous species, we included them in this study because of their potential ability to modulate GSH metabolism: we have previously shown that they increase GSH levels in *Arabidopsis* (May et al., in preparation). Samples were collected 0, 3, and 27 h after the addition of the chemicals, and steady-state levels of *AtMRP1*, *AtMRP3*, *AtMRP4*, and γ -ECS genes were determined by Northern analysis. As in the previous experiments, *AtMRP3* was the most inducible member of the *AtMRP* gene family: its mRNA levels increased more than fourfold after 27 h of treatment with benoxacor or oxabectrinil (both GST inducers in monocotyledonous species). Cloquintocet, fenclorazole, and flurazone had a moderate effect on the levels of *AtMRP3* mRNA within 3 h of treatment, but levels did not change at later times (Fig. 5). In contrast, both *AtMRP1* and *AtMRP4* showed a moderate and reversible induction peaking after 3 h of treatment with all safeners except oxabectrinil (Fig. 5).

From these expression analyses, we can conclude that *AtMRP1* is the gene that is least responsive to the treatments used, whereas *AtMRP3* is the most inducible. *AtMRP4* lies between these extremes, being induced by certain treatments but never reaching the levels of induction shown by *AtMRP3*. These data are in agreement with those described by Tommasini et al. (1997), who showed that treatments of whole *Arabidopsis* plants with different xenobiotic compounds produced a marked induction of *AtMRP3*, whereas *AtMRP1* mRNA levels were unaffected and *AtMRP4* showed moderate induction.

It is important to note that both *AtMRP3* and *AtMRP4*, but not *AtMRP1*, seem to be induced under oxidative stress conditions, revealing that the structural classification based on sequence comparison (Fig. 2) may reflect functional similarities. However, we cannot rule out the possibility that aminotriazole and/or menadione may be affecting the expression of the different members of the *AtMRP* gene family through some non-

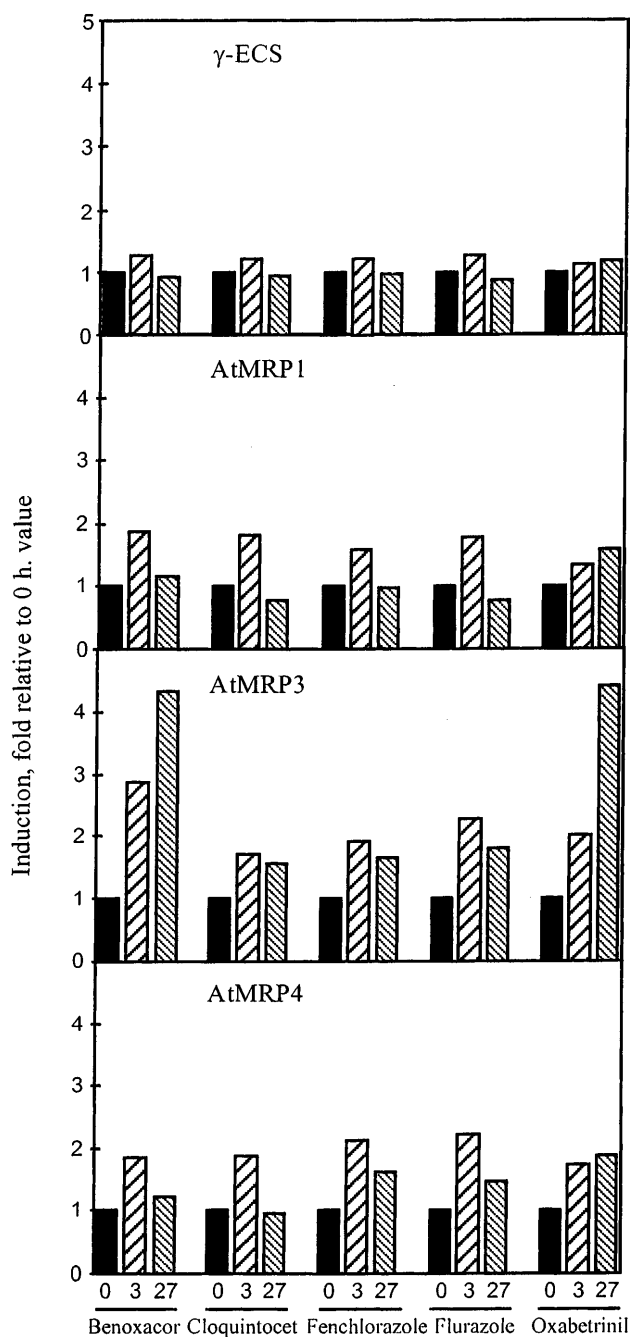


Fig. 5 Changes in *AtMRP1*, *AtMRP3*, and *AtMRP4* mRNA levels as quantified by phosphor-imaging after treatment of cell suspensions for 0, 3, and 27 h with herbicide safeners. All compounds were used at a final concentration of 10 μ M. As a constitutive control, the levels of expression of the γ -ECS gene, encoding the first enzyme in the biosynthesis of GSH, are shown

specific mechanism not directly linked to oxidative stress. In fact, aminotriazole has been found to inhibit histidine and carotenoid biosynthesis in some organisms. Even though it has been shown that aminotriazole does not exert its toxic effect in whole *Arabidopsis* plants through inhibition of amino acid or carotenoid biosynthesis (Heim and Larrinua 1989), we cannot eliminate completely the possibility that aminotriazole has some

secondary effect on those pathways in *Arabidopsis* cell suspensions that could play a role in the generation of the gene induction pattern described.

In the light of the results presented here, it will be of particular interest to determine the substrate specificities and affinities of the different members of the AtMRP family. In order to extend these observations, it will be necessary to carry out whole-plant studies and also to perform in situ hybridizations using the probes available for the different *AtMRP* genes. This will allow us to determine whether differences in the spatial distribution and/or substrate specificity of these proteins exist, facilitating prediction of their physiological function.

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