Volume 6 number 9 September 1987

THE EMBO JOURNAL



The EMBO MEDAL 1987

Published monthly for
the European Molecular Biology Organization
by IRL Press

Corteva Exhibit 2059 - Page 1 of 11
Inari v. Corteva - PGR2023-00022

THE EMBO JOURNAL

Editor in chief
Max Birnstiel, *Chairman of EMBO Council*

Executive editor
John Tooze, *Executive Secretary of EMBO*

Editorial board
F.Amaldi, *Rome, Italy (1987)*
K.Beyreuther, *Köln, FRG (1987)*
F.Blasi, *Copenhagen, Denmark (1985)*
P.Cohen, *Dundee, UK (1985)*
R.Cortese, *EMBL, Heidelberg, FRG (1985)*
L.Crawford, *London, UK (1985)*
F.Cuzin, *Nice, France (1987)*
J.Drenth, *Groningen, The Netherlands (1986)*
J.Engel, *Basel, Switzerland (1985)*
A.Fersht, *London, UK (1987)*
W.Franke, *Heidelberg, FRG (1985)*
D.Gallwitz, *Göttingen, FRG (1987)*
M.Hofnung, *Paris, France (1985)*
B.Hohn, *Basel, Switzerland (1986)*
D.Hopwood, *Norwich, UK (1986)*
B.Jordan, *Marseille, France (1985)*
T.Jovin, *Göttingen, FRG (1986)*
W.Keller, *Heidelberg, FRG (1986)*
F.Lacroute, *Strasbourg, France (1985)*
P.Lawrence, *Cambridge, UK (1987)*
C.Leaver, *Edinburgh, UK (1985)*
D.Lilley, *Dundee, UK (1986)*
D.Louvard, *Paris, France (1986)*
K.Marcker, *Aarhus, Denmark (1985)*
J.Meldolesi, *Milan, Italy (1986)*
M.Melli, *Siena, Italy (1987)*
M.van Montagu, *Gent, Belgium (1986)*
K.Nasmyth, *Cambridge, UK (1987)*
W.Neupert, *München, FRG (1986)*
K.Nordström, *Uppsala, Sweden (1986)*
S.Numa, *Kyoto, Japan (1987)*
V.Pirrotta, *Houston, TX, USA (1986)*
R.Planta, *Amsterdam, The Netherlands (1987)*
D.Richter, *Hamburg, FRG (1987)*
J.-D.Rochaix, *Geneva, Switzerland (1986)*
J.Schlessinger, *Rockville, MD, USA (1987)*
G.Schütz, *Heidelberg, FRG (1985)*
J.Seelig, *Basel, Switzerland (1986)*
J.P.Thiery, *Nogent-sur-Marne, France (1987)*
H.Thoenen, *Martinsried, FRG (1985)*
R.Timpl, *Martinsried, FRG (1985)*
M.Wikström, *Helsinki, Finland (1987)*
M.Yaniv, *Paris, France (1986)*
R.Zinkernagel, *Zürich, Switzerland (1987)*

Production staff
Stuart Hill, *production editor, IRL Press*
Suzanne M.Evans, *editorial assistant, IRL Press*

Published for the European Molecular Biology Organization, Postfach 1022.40, 6900 Heidelberg 1, FRG, by IRL Press Ltd, PO Box 1, Eynsham, Oxford OX8 1JJ, UK

EMBO
IRL PRESS
OXFORD WASHINGTON DC

Contents

The EMBO Journal
Volume 6 number 9 September 1987

THE EMBO MEDAL REVIEW

Clathrin and coated vesicles — B.M.F.Pearse — 2507

ORIGINAL PAPERS

Engineering herbicide resistance in plants by expression of a detoxifying enzyme — M.De Block, J.Botterman, M.Vandewiele, J.Dockx, C.Thoen, V.Gosselé, N.Rao Movva, C.Thompson, M.Van Montagu and J.Leemans — 2513

Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus* — C.J.Thompson, N.Rao Movva, R.Tizard, R.Crameri, J.E.Davies, M.Lauwereys and J.Botterman — 2519

Cloning of a *Nicotiana plumbaginifolia* protoplast-specific enhancer-like sequence — M.Horth, I.Negrutiu, A.Burny, M.Van Montagu and L.Herrera-Estrella — 2525

Metal ion regulated gene expression: use of a plastocyanin-less mutant of *Chlamydomonas reinhardtii* to study the Cu(II)-dependent expression of cytochrome *c*-552 — S.Merchant and L.Bogorad — 2531

The 5′-proximal region of the wheat Cab-1 gene contains a 268-bp enhancer-like sequence for phytochrome response — F.Nagy, M.Bouty, M.-Y.Hsu, M.Wong and N.-H.Chua — 2537

Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the rbcS-3A gene — P.J.Green, S.A.Kay and N.-H.Chua — 2543

Parsley protoplasts retain differential responsiveness to u.v. light and fungal elicitor — J.L.Dangl, K.D.Hauffe, S.Lipphardt, K.Hahlbrock and D.Scheel — 2551

Function of the 30 kd protein of tobacco mosaic virus: involvement in cell-to-cell movement and dispensability for replication — T.Meshi, Y.Watanabe, T.Saito, A.Sugimoto, T.Maeda and Y.Okada — 2557

Organization of the fibronectin gene provides evidence for exon shuffling during evolution — R.S.Patel, E.Odermatt, J.E.Schwarzbauer and R.O.Hynes — 2565

Multiple sites of alternative splicing of the rat fibronectin gene transcript — J.E.Schwarzbauer, R.S.Patel, D.Fonda and R.O.Hynes — 2573

Characterization of the cell adhesion molecules L1, N-CAM and J1 in the mouse intestine — G.Thor, R.Probstmeier and M.Schachner — 2581

Characterization of a slowly proliferative cell along the oligodendrocyte differentiation pathway — M.Dubois-Dalcq — 2587

Microtubules containing detyrosinated tubulin are less dynamic — T.E.Kreis — 2597

Analysis of cytokeratin domains by cloning and expression of intact and deleted polypeptides in *Escherichia coli* — T.M.Magin, M.Hatzfeld and W.W.Franke — 2607

The nuclear migration signal of *Xenopus laevis* nucleoplasmin — T.R.Bürglin and E.M.De Robertis — 2617

Mitochondrial porin of *Neurospora crassa*: cDNA cloning, *in vitro* expression and import into mitochondria — R.Kleene, N.Pfanner, R.Pfaller, T.A.Link, W.Sebald, W.Neupert and M.Tropschug — 2627

High-affinity binding sites involved in the import of porin into mitochondria — R.Pfaller and W.Neupert — 2635

Corteva Exhibit 2059 - Page 3 of 11
Inari v. Corteva - PGR2023-00022

Contents (continued)

The EMBO Journal
Volume 6 number 9 September 1987

The influenza hemagglutinin precursor as an acid-sensitive probe of the biosynthetic pathway	F.Boulay, R.W.Doms, I.Wilson and A.Helenius	**2643**
Functional reconstitution of influenza virus envelopes	T.Stegmann, H.W.M.Morselt, F.P.Booy, J.F.L.van Breemen, G.Scherphof and J.Wilschut	**2651**
Phosphorylation of the human transferrin receptor by protein kinase C is not required for endocytosis and recycling in mouse 3T3 cells	M.Zerial, M.Suomalainen, M.Zanetti-Schneider, C.Schneider and H.Garoff	**2661**
An insertional mutant of epidermal growth factor receptor allows dissection of diverse receptor functions	E.Livneh, N.Reiss, E.Berent, A.Ullrich and J.Schlessinger	**2669**
M_r 46 000 mannose 6-phosphate specific receptor: its role in targeting of lysosomal enzymes	M.Stein, J.E.Zijderhand-Bleekemolen, H.Geuze, A.Hasilik and K.von Figura	**2677**
Deletion analysis of the internal signal–anchor domain of the human asialoglycoprotein receptor H1	M.Spiess and C.Handschin	**2683**
Human CSF-1: gene structure and alternative splicing of mRNA precursors	M.B.Ladner, G.A.Martin, J.A.Noble, D.M.Nikoloff, R.Tal, E.S.Kawasaki and T.J.White	**2693**
Coordinate expression of the endogenous p53 gene in β cells of transgenic mice expressing hybrid insulin–SV40 T antigen genes	S.Efrat, S.Baekkeskov, D.Lane and D.Hanahan	**2699**
Retroviral expression of the human IL-2 gene in a murine T cell line results in cell growth autonomy and tumorigenicity	G.Yamada, Y.Kitamura, H.Sonoda, H.Harada, S.Taki, R.C.Mulligan, H.Osawa, T.Diamantstein, S.Yokoyama and T.Taniguchi	**2705**
Identification and purification of a polypeptide that binds to the c-*fos* serum response element	R.Treisman	**2711**
The highly conserved amino-terminal region of the protein encoded by the v-*myb* oncogene functions as a DNA-binding domain	K.-H.Klempnauer and A.E.Sippel	**2719**
Regulation of pp56^{lck} during T-cell activation: functional implications for the *src*-like protein tyrosine kinases	J.D.Marth, D.B.Lewis, C.B.Wilson, M.E.Gearn, E.G.Krebs and R.M.Perlmutter	**2727**
Ig gene rearrangement and expression in the progeny of B-cell progenitors in the course of clonal expansion in bone marrow cultures	N.Yoshida, A.Radbruch and K.Rajewsky	**2735**
Differences in B cell growth phenotype reflect novel patterns of Epstein–Barr virus latent gene expression in Burkitt's lymphoma cells	M.Rowe, D.T.Rowe, C.D.Gregory, L.S.Young, P.J.Farrell, H.Rupani and A.B.Rickinson	**2743**
Polymeric immunoglobulin M is secreted by transfectants of non-lymphoid cells in the absence of immunoglobulin J chain	A.Cattaneo and M.S.Neuberger	**2753**
***Cis*- and *trans*-acting elements responsible for the cell-specific expression of the human α1-antitrypsin gene**	V.De Simone, G.Ciliberto, E.Hardon, G.Paonessa, F.Palla, L.Lundberg and R.Cortese	**2759**
The human α_1-antitrypsin gene is transcribed from two different promoters in macrophages and hepatocytes	E.Perlino, R.Cortese and G.Ciliberto	**2767**
Coincident start sites for divergent transcripts at a randomly selected CpG-rich island of mouse	P.Lavia, D.Macleod and A.Bird	**2773**
GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA	I.A.Hope and K.Struhl	**2781**

Corteva Exhibit 2059 - Page 4 of 11
Inari v. Corteva - PGR2023-00022

Contents (continued)

The EMBO Journal
Volume 6 number 9 September 1987

Decapentaplegic transcripts are localized along the dorsal–ventral axis of the *Drosophila* embryo	R.D.St.Johnston and W.M.Gelbart	**2785**
The *gooseberry–zipper* region of *Drosophila:* five genes encode different spatially restricted transcripts in the embryo	S.Côté, A.Preiss, J.Haller, R.Schuh, A.Kienlin, E.Seifert and H.Jäckle	**2793**
The transcription unit of the *Drosophila engrailed* locus: an unusually small portion of a 70 000 bp gene	B.Drees, Z.Ali, W.C.Soeller, K.G.Coleman, S.J.Poole and T.Kornberg	**2803**
A cell-free plant extract for accurate pre-tRNA processing, splicing and modification	N.Stange and H.Beier	**2811**
Structural models of the redox centres in cytochrome oxidase	L.Holm, M.Saraste and M.Wikström	**2819**
Isolation and analysis of the genes for cytochrome *c* oxidase in *Paracoccus denitrificans*	M.Raitio, T.Jalli and M.Saraste	**2825**
Release of a chimeric protein into the medium from *Escherichia coli* using the C-terminal secretion signal of haemolysin	N.Mackman, K.Baker, L.Gray, R.Haigh, J.-M.Nicaud and I.B.Holland	**2835**
The pAR5 mutation and the allosteric mechanism of *Escherichia coli* aspartate carbamoyltransferase	J.Cherfils, P.Vachette, P.Tauc and J.Janin	**2843**
Construction of hybrid Tn*501*/Tn*21* transposases *in vivo*: identification of a region of transposase conferring specificity of recognition of the 38-bp terminal inverted repeats	L.R.Evans and N.L.Brown	**2849**
Author index		**2855**
Errata		**2856**
Product news		
Situations vacant/announcements		

Cover illustration: The cover shows engineered and control tomato plants sprayed with high doses of the commercial herbicide BASTA® (20 l/ha). For more details, see pages 2513–2518 and 2519–2523 in this issue.

The EMBO Journal vol.6 no.9 pp.2513–2518, 1987

Engineering herbicide resistance in plants by expression of a detoxifying enzyme

M.De Block, J.Botterman, M.Vandewiele, J.Dockx, C.Thoen, V.Gosselé, N.Rao Movva[1], C.Thompson[1,2], M.Van Montagu and J.Leemans

Plant Genetic Systems N.V., J.Plateaustraat 22, B-9000 Gent, Belgium, and [1]Biogen S.A., 46, Route des Acacias, CH-1227 Geneva, Switzerland

[2]Present address: Institut Pasteur, 25 et 28 Rue du Docteur Roux, 75724 Paris F-Cedex 15, France

Communicated by M.Van Montagu

Phosphinothricin (PPT) is a potent inhibitor of glutamine synthetase in plants and is used as a non-selective herbicide. The *bar* gene which confers resistance in *Streptomyces hygroscopicus* to bialaphos, a tripeptide containing PPT, encodes a phosphinothricin acetyltransferase (PAT) (see accompanying paper). The *bar* gene was placed under control of the 35S promoter of the cauliflower mosaic virus and transferred to plant cells using *Agrobacterium*-mediated transformation. PAT was used as a selectable marker in protoplast co-cultivation. The chimeric *bar* gene was expressed in tobacco, potato and tomato plants. Transgenic plants showed complete resistance towards high doses of the commercial formulations of phosphinothricin and bialaphos. These data present a successful approach to obtain herbicide-resistant plants by detoxification of the herbicide.

Key words: acetyltranferase/bialaphos/herbicide resistance/glutamine synthetase/phosphinothricin

Introduction

The use of herbicides to reduce loss in crop yield due to weeds has become an integral part of modern agriculture. There is a continuous search for new herbicides that are highly effective and safe for animals and the environment. A new class of herbicides that fulfils these needs acts by inhibiting specific amino acid biosynthesis pathways in plants (La Rossa and Falco, 1984). However, most of these herbicides do not distinguish between weeds and crops.

Modifying plants to become resistant to such broad-spectrum herbicides would allow their selective use for crop protection. As a consequence, a major effort has been devoted in several laboratories to engineer herbicide-resistant plants. Two approaches have been followed. In the first, a mutant form of the target enzyme is produced which is still active but less sensitive to the herbicide. In this way, mutant plants producing an altered form of the enzyme acetolactate synthase have been selected which are resistant to the sulfonylurea and imidazolinone herbicides (Chaleff and Ray, 1984; Shaner and Anderson, 1985). In another example, a mutant form of the bacterial *aro*A gene was expressed in tobacco and conferred tolerance to the herbicide glyphosate (Comai *et al.*, 1985). The second approach involves overproduction of the target enzyme. It has been demonstrated that overexpression of the plant enzyme 5-enol-pyruvylshikimate-3 phosphate synthase conferred glyphosate tolerance in transgenic petunia plants (Shah *et al.*, 1986).

Bialaphos (Ogawa *et al.*, 1973) and phosphinothricin (PPT) (Bayer *et al.*, 1972) are potent new herbicides. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus*. It consists of PPT, an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of these residues by peptidases, PPT is a potent inhibitor of glutamine synthetase (GS). This enzyme plays a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants (Miflin and Lea, 1977; Skokut *et al.*, 1978). It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation and photorespiration. Inhibition of GS by PPT causes rapid accumulation of ammonia which leads to death of the plant cell (Tachibana *et al.*, 1986). PPT is chemically synthesized (Basta®, Hoechst AG) while bialaphos is produced by fermentation of *S. hygroscopicus* (Herbiace®, Meiji Seika Ltd). Here we present an alternative strategy to engineer herbicide resistance in plants by expressing an enzyme that detoxifies phosphinothricin.

The accompanying paper describes the cloning and characterization of a bialaphos resistance gene (*bar*) from *S. hygroscopicus* which is involved in the bialaphos biosynthesis pathway. It encodes a phosphinothricin acetyltransferase (PAT), which acetylates the free NH_2 group of PPT and thereby prevents autotoxicity in the producing organism (Murakami *et al.*, 1986). Here we report the expression of the *bar* gene in transgenic tobacco, tomato and potato plants. We show that transgenic plants expressing PAT are completely resistant to high doses of the commercial formulations of both PPT and bialaphos. The use of the *bar* gene as reporter gene to analyse plant gene expression is also demonstrated.

Results

Chimeric bar gene for expression in plants

The *bar* gene was originally isolated in a streptomycete vector and subcloned into an *Escherichia coli* vector yielding pBG195 (accompanying paper). The translation initiation codon of the *bar* gene in *Streptomyces* is GTG. In order to guarantee proper translation initiation in plants, an ATG initiation codon is required. To this end, the N-terminal end of the *bar* coding region was substituted for two complementary synthetic oligonucleotides (Figure 1A). In the resulting plasmid, pGSFR1, the *bar* gene contains an ATG as initiation codon. Subsequently, this gene was inserted between the cauliflower mosaic 35S promoter and the termination and polyadenylation signal of the octopine T-DNA gene 7 (Velten and Schell, 1985). This chimeric gene and a kanamycin resistance gene under the control of the nopaline synthase promoter were inserted between the octopine T-DNA borders of plant transformation vector pGV1500 (Figure 1B). This plasmid, pGSFR280, was mobilized into the *Agrobacterium* recipient C58C1 Rif^R (pGV2260) (Deblaere *et al.*, 1985), to generate strain C58C1 Rif^R (pGSFR1280).

PAT is a selectable marker in tobacco protoplast transformation

To test whether expression of the PAT enzyme allows for selec-

© IRL Press Limited, Oxford, England

Corteva Exhibit 2059 - Page 6 of 11
Inari v. Corteva - PGR2023-00022

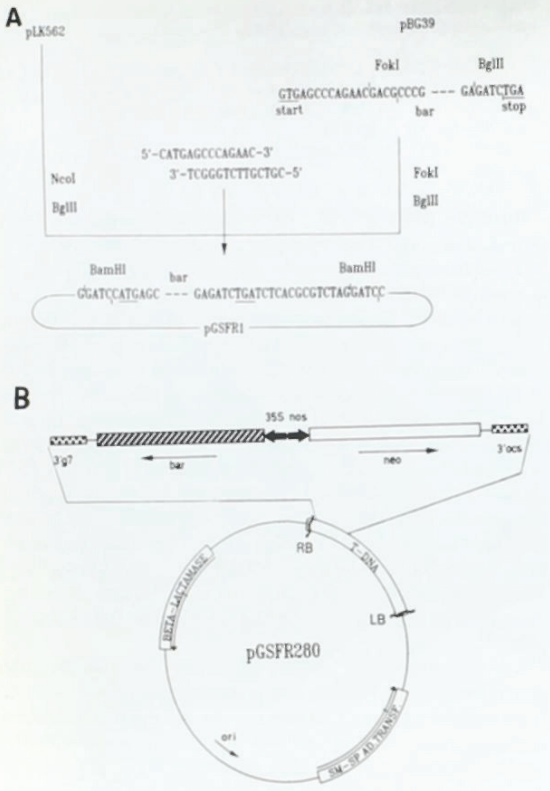


Fig. 1. (**A**) Construction of a *bar* gene cassette. pGSFR1 contains an intact *bar* gene with an ATG initiation codon. The nucleotide sequence of the 5' and 3' end of the gene are shown and the initiation and stop codon are underlined. (**B**) Schematic representation of pGSFR280. Both chimeric genes, the *bar* gene under control of the CaMV 35S promoter and the *neo* gene under control of the *nos* promoter, are inserted in divergent orientation between the T-DNA border repeats of pGV1500 (RB: right border and LB: left border). The respective genes are followed by fragments encoding termination and polyadenylation signals of ocs (3'ocs) and T-DNA gene 7 (3'g7).

tion of transformed plant cells, leaf protoplasts of *Nicotiana tabacum* cv. Petit Havana (SR1) were co-cultivated with the *Agrobacterium* strains C58C1 Rif^R (pGSFR1280). C58C1 Rif^R (pGV2260) was used as a control strain that lacks the *bar* gene. Transformants were selected in medium containing 50 mg/l kanamycin or various concentrations of PPT (0.5−50 mg/l). After 1 month, no growth was observed on the selective plates of the C58C1 Rif^R (pGV2260) co-cultivation. Thus, the low dose of 0.5 mg/l PPT is sufficient to inhibit growth of sensitive cells. Calli transformed with C58C1 Rif^R (pGSFR1280) grew indistinguishable from their growth in non-selective medium under all selective conditions. Twenty calli that had been selected on 50 mg/l PPT were then grown for 2 months on medium containing 50 mg/l PPT. Subsequently they were subcultured on media containing different levels of PPT ranging from 50 to 1000 mg/l. All calli grew on medium containing 500 mg/l PPT. At a concentration of 1000 mg/l of PPT, their growth rate was slightly reduced, and three out of the 20 calli died after a few weeks.

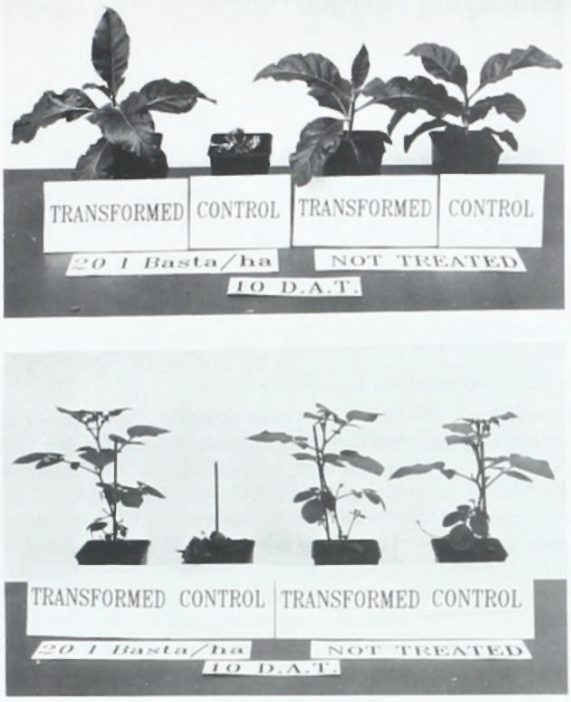


Fig. 2. Herbicide resistance in transgenic tobacco plants (*N. tabacum* cv. Petit Havana SR1 plants) (**A**) and transgenic potato plants (*S. tuberosum* cv. Berolina). (**B**) Untransformed control and transformed plants were sprayed with 20 l Basta® /ha. A control series of plants was not treated. The treated control plant was killed within 10 days, while the treated transformed plant grew as well as the untreated control.

Transgenic plants are fully resistant to the herbicides Basta® and Herbiace®

N. tabacum cv. Petit Havana SR1 plants expressing the *bar* gene were obtained by the leaf disc infection method (Horsch *et al.*, 1985). Transformed shoots were selected after infection with C58C1Rif^R (pGSFR1280) on medium containing either 50 mg/l kanamycin or 1−50 mg/l PPT. Leaf discs infected with control strain C58C1Rif^R (pGV2260) bleached within 1 week on PPT-containing medium.

We then evaluated whether expression of PAT confers resistance to commercial formulations of PPT and bialaphos. Transgenic plants were transferred to soil and grown in the greenhouse. Their growth was indistinguishable from untransformed control plants. Control SR1 and transgenic plants were sprayed with doses equivalent to 8 l/ha and 20 l/ha Basta® . Basta® at 2 l/ha effectively kills control SR1 plants in 10 days. All 21 of the transgenic plants which were assayed were fully resistant to the herbicide (Figure 2). Two additional applications of the herbicide within a 4-week period did not affect growth of the plants. Treated plants flowered normally and set seed. Transgenic plants were also sprayed with 8 l/ha and 20 l/ha of Herbiace® , the commercial formulation of bialaphos. They also proved to be fully resistant to these applications.

Ammonia levels in herbicide-treated plants

As a more sensitive indicator of GS inhibition, we have measured

Corteva Exhibit 2059 - Page 7 of 11
Inari v. Corteva - PGR2023-00022

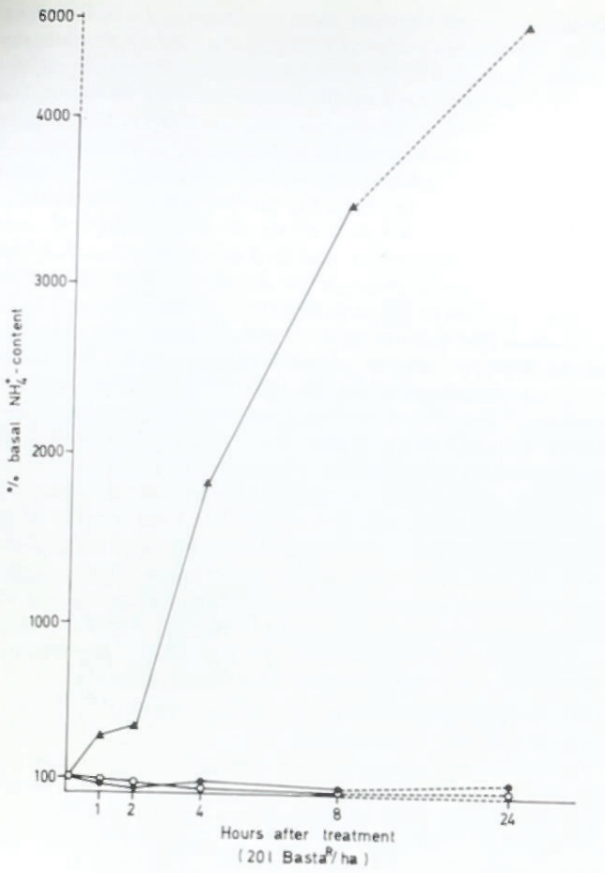


Fig. 3. Ammonia determination (% basal NH_4^+ content) in transformed and untransformed tobacco plants after spraying with 20 l Basta® /ha. Samples were taken before spraying and at regular time points after spraying. ○ untreated control plant; ● treated transgenic plant; ▲ treated control plant.

ammonia accumulation in transgenic and non-transformed plants treated with the herbicide. Ammonia accumulated rapidly in treated control plants and increased 40-fold after 8 h. Ammonia levels in transgenic plants did not significantly change over a 24-h period after application of 20 l Basta®/ha. The levels were comparable to those present in untreated SR1 plants (Figure 3). This clearly shows that the transgenic plant is completely insensitive to the herbicide treatment.

Inheritance of PPT resistance

Seven transgenic plants that had been treated with 20 l/ha Basta® were analysed further (plants 101–105, 107 and 108). They produced normal amounts of viable seed. Five week old plants from the F1 progeny were sprayed with 8 l/ha Basta® . From the seven parental plants, six segregated resistant and sensitive seedlings in a 3 to 1 ratio, indicating Mendelian inheritance as a single dominant locus. Southern blots showed that 103 contained three copies of *bar*, whereas the others carried a single copy (data not shown). Since a Mendelian inheritance was observed, we expect that the three T-DNA copies are inserted at a single locus in plant 103. One plant (101) did not produce resistant seedlings. Also no PAT enzyme activity could be detected in these seedlings. This observation needs further investigation.

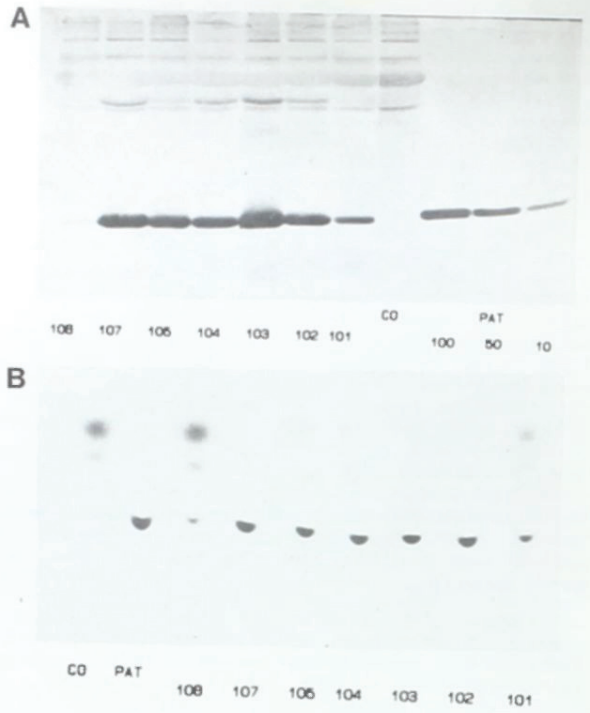


Fig. 4. (**A**) Detection of PAT protein by immunoblotting of leaf extracts from transgenic *N. tabacum* plants. Crude extracts of 50 μg protein were separated on an SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. The filter was treated with PAT antiserum and stained. **Lanes 1–7**, crude extracts from seven independent plants. **Lane 8** crude extract from an untransformed plant (CO). **Lanes 9–11**, 100, 50 and 10 ng of purified PAT. (**B**) Detection of PAT activity by t.l.c. The extracts were prepared from leaf tissue and diluted 100 times. The reaction was performed and an aliquot spotted on a silicagel t.l.c. plate for ascendent chromatography. [^{14}C]Substrates and reaction products were visualized by autoradiography. **Lane 1**, leaf extract from untransformed plant. **Lane 2**, leaf extract of untransformed plant to which PAT enzyme is added. **Lanes 3–9**, extracts from seven independent transgenic tobacco plants.

Expression of PAT in transgenic plants

The expression level of PAT was determined in the seven selected transformed tobacco plants. Analysis of crude leaf extracts by Western blotting revealed the synthesis of a polypeptide that co-migrated with PAT, purified from an *E. coli* overproducing strain (in preparation) (Figure 4A). Plants 101 and 108 produce low levels of PAT (<0.01%), whereas it amounts up to 0.1% of total soluble protein in the others. The same relative levels of PAT activity were observed in an enzymatic assay in which ^{14}C-labelled acetylated PPT was detected after separation by t.l.c. (Figure 4B). Using a spectrophotometric assay which is more convenient for quantification, no activity above the background level could be measured in extracts of 108, whereas a 100-fold dilution of the extract of 103 still contained detectable activity (Table I). Interestingly, the low level expression in 108 was still sufficient to fully protect the plant from the herbicide.

PAT-specific mRNA was detected in a Northern blot of total RNA extracted from young leaves of the plants (Figure 5). Although all of these plants contain the same gene, they showed at least a 100-fold variation in the accumulation of PAT-specific mRNA as assayed by autoradiography densitometry. In the different plants the ratio in the levels of steady-state mRNA corre-

Corteva Exhibit 2059 - Page 8 of 11
Inari v. Corteva - PGR2023-00022

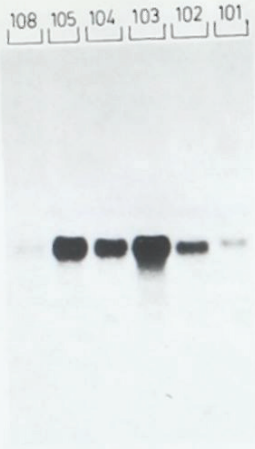


Fig. 5. Northern blot hybridization of RNA extracted from six transgenic tobacco plants. 10 μg aliquots of total RNA were electrophoresed in 1.5% agarose/formaldehyde gel and blotted to nitrocellulose. A *bar* RNA probe was used for hybridization.

Table I. PAT activity in crude leaf extracts in transgenic tobacco plants measured spectrophotometrically

Plants	Activity (10^{-6} units/mg extracted protein)
108	n.d.
107	704
105	264
104	304
103	1240
102	407
101	43

Enzyme assay and definition of unit activity are described in Materials and methods. n.d. = not detectable

sponded to the amount of PAT protein accumulated (compare Figure 5 and Figure 4A).

Herbicide-resistant tomato and potato plants

Transgenic plants expressing PAT were obtained from leaf disc infections of *Lycopersicum esculentum* cv. Lukullus and *Solanum tuberosum* cv. Berolina, cv. Bintje and cv. Désiree. The two tomato and three potato plants, which were sprayed with 20 l/ha Basta® , were fully resistant (Figure 2B). All the transgenic plants tested (15 tomatoes and 26 potatoes) showed high levels of PAT activity.

Discussion

The *bar* gene from *S. hygroscopicus* encodes a PAT which converts PPT into the non-toxic acetylated form (accompanying paper). We have used *Agrobacterium* vectors to transfer the *bar* gene in tobacco, tomato and potato plants. When expressed from the constitutive cauliflower mosaic virus 35S promoter an active PAT enzyme was produced in the plant cells. Transgenic plants were completely resistant towards the herbicide. We also demonstrated the use of *bar* gene as a convenient selectable and screenable marker in plant transformation experiments.

Low doses of 0.5 mg/l PPT are sufficient to inhibit growth of tobacco protoplasts. The *bar* gene proved to be an efficient dominant selectable marker in tobacco protoplast transformation. Tobacco calli expressing the PAT enzyme grew on medium containing 500 mg/l PPT. It was also possible to select directly for transformed shoots in leaf disc transformations. Similar results were obtained with chimeric constructs carrying the *bar* gene under control of other plant promoters (unpublished data).

The *bar* gene is a useful reporter gene. It is enzymatically and immunologically assayable to analyse gene expression in transformed plants. In Western blots, amounts of 5 ng PAT were detectable (Figure 3A). In addition, its enzymatic activity can be analysed by chromatographic detection of acetylated PPT (Figure 3B). Enzymatic activities corresponding to 1 ng of protein are clearly observed after an overnight exposure. Accurate values of enzyme activity are obtained if the enzyme kinetics are analysed spectrophotometrically (see Table I). Enzyme concentrations of 5 ng/ml can be detected above background acetylase activity in plant extracts. Enzymatic characterization of PAT and some derivatives will be described elsewhere.

Analysis of the PAT expression demonstrated that there is a substantial variation between independent transformants in the levels of expression, as has been observed in other cases (Jones *et al.*, 1985; Sanders *et al.*, 1987). Quantitative data of activities showed at least a 30-fold difference. This variability between different plants was represented at the level of PAT synthesis, PAT activity and the amount of PAT-specific mRNA. The presence of three T-DNA copies in plant 103 (data not shown) may explain the highest expression level; the other observed differences may be due to chromosomal location of the T-DNA insertion.

In greenhouse spraying tests transgenic plants expressing various levels of PAT were fully resistant to high doses of Basta® and Herbiace® , the commercial formulations of PPT and bialaphos, respectively. For example, Basta® effectively kills tobacco plants at 2 l/ha while transgenic plants were fully resistant to repeated spraying with 20 l/ha (Figure 2). Normal applications for weed control in agriculture vary from 2.5 to 7.5 l/ha. The fact that treated resistant plants did not show any increase in NH_4^+ content proved the complete protection of the plant GS from the action of the herbicide. Expression of PAT at the level of 0.001% of total extracted proteins is sufficient to protect the plants against field-dose application of the herbicides. Some plants express PAT at a 100-fold higher level. The PPT resistance was inherited in the F1 progeny of tobacco as a single dominant trait. Spraying of the seedlings can be used to follow segregation. Thus, the *bar* gene offers a great advantage for selection of progeny grown in soil; this in contrast with many other marker genes.

Other approaches to obtain PPT-resistant plants have been proposed. Donn *et al.* (1984) selected PPT-tolerant alfalfa cell suspensions. These resulted from gene amplification which yielded an increase in the GS expression. A similar result can likely be obtained by overexpressing a GS gene, using a strong plant promoter. However, it is not clear whether converting more glutamate into glutamine would not produce undesired effects on the nitrogen metabolism of the plant (Loyola-Vargas and Sanchez de Jimenez, 1984). Another approach would be to introduce into plants a mutant form of GS which is no longer inhibited by PPT. Since plant GS can complement a *gln* A mutation in *E. coli* (Das Sarma *et al.*, 1986), it might be possible to select directly in *E. coli* for mutant forms of the plant GS which are resistant to PPT. However, severe drawbacks could be encountered if the resistant GS has modified enzymatic properties. Also, GS has a multimeric subunit structure and mixed enzyme complexes will probably be inhibited by the herbicide. Furthermore, it is

Corteva Exhibit 2059 - Page 9 of 11
Inari v. Corteva - PGR2023-00022

not clear whether overexpression or mutation of a single GS form would result in herbicide resistance since many plants contain multiple forms of GS (Ericson, 1985; Gebhardt *et al.*, 1986).

Our results clearly show that engineering herbicide resistance using a detoxification or degradation process holds much promise. The successful engineering of a detoxification pathway will be largely independent from the plant species used. The same gene will thus be useful to engineer a variety of crops, as we have exemplified here. There are two sources for detoxifying enzymes in nature; some herbicide-tolerant crop species have detoxifying pathways such as the glutathione-S-transferase in corn (Shimabukuro *et al.*, 1971). Another source can be degradative microorganisms often found in herbicide-contaminated soils. Problems with this approach could arise if several enzymes are involved in the detoxification or if the enzymes have only a low affinity and/or specificity for the herbicides. The advantages of PAT as described here are in part the consequence of its evolution as resistance gene to act very efficiently and with high specificity on phosphinothricin.

Materials and methods

Plasmid constructions

All DNA manipulations were as described by Maniatis *et al.* (1982). A *Fok*I–*Bgl*II fragment was isolated from pBG39 (accompanying paper) and annealed with two complementary synthetic oligonucleotides 5′-CATGAGCCCAGAAC-3′ and 5′-CGTCGTTCTGGGCT-3′ and ligated to pLK562 (Botterman, 1986) which had been digested with *Nco*I and *Bgl*II. The *bar* gene could be isolated as a *Bam*HI fragment from the resulting plasmid pGSFR1. A cauliflower mosaic virus 35S promoter fragment, obtained from pGSJ280 (Deblaere *et al.*, 1987) was fused at the *Cla*I site to the *Bam*HI fragment containing the *bar* gene, after filling the protruding ends with Klenow DNA polymerase. The TL-DNA gene 7, isolated from pAP2034, was added as the 3′g7 fragment (Velten and Schell, 1985).

A chimeric kanamycin resistance marker containing a nopaline synthase promoter, the neomycin phosphotransferase II gene from *Tn*5 and the 3′ end of the octopine synthase gene was inserted in the *Bgl*II site of pGV1500 (Deblaere *et al.*, 1987). Subsequently, the chimeric *bar* gene was inserted in this vector to generate pGSFR280.

Transformation of regenerating SR1 tobacco protoplasts by co-cultivation with Agrobacterium tumefaciens

Leaf protoplasts of *N. tabacum* c.v. Petit havana (SR1) (Maliga *et al.*, 1973) were isolated from sterile shoot cultures grown on medium containing half the concentration of MS salts (Murashige and Skoog, 1962), supplemented with 1% sucrose, 0.8% agar, pH 5.7. SR1 tobacco protoplasts were transformed using a modification of the co-cultivation technique of Marton *et al.* (1979). Five ml of protoplasts ($\sim 10^5$/ml) were cultured in K3 medium (Nagy and Maliga, 1976), supplemented with 0.4 M sucrose, 0.5 g/l MES, pH 5.5, 0.1 mg/l naphthaleneacetic acid (NAA) and 0.2 mg/l benzylaminopurine (BAP) in 9 cm Petri dishes for 3 days. Regenerating protoplasts were then infected at a ratio of ~100 bacterial cells, grown in Min A medium (Miller, 1972) per protoplast. After 3 days, 2.5 ml of modified K3 medium containing 1.2% low gelling agarose and 1000 mg/l cefotaxime was added to 2.5 ml of the co-cultivation mixture. The culture was incubated in 5 cm Petri dishes. One week later the agarose discs were transferred to 9 cm Petri dishes containing 10 ml of modified K3 medium with 0.35 M sucrose and 50 mg/l kanamycin or 0.5–50 mg/l PPT. Every 5 days the liquid medium was replaced by fresh medium, each time lowering the sucrose concentration by 50 mM. When the sucrose concentration reached 0.1 M, the calli were transferred to B5 medium (Gamborg, 1968) with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.7, 0.2 mg/l BAP, 0.6 mg/l NAA, 0.6% agarose and supplemented with various concentrations of kanamycin or PPT.

Leaf disc transformation

Tobacco. Leaves from sterile shoot cultures of *N. tabacum* cv. Petit Havana SR1, grown on medium containing half the concentration of MS salts supplemented with 1% sucrose, 0.8% agar, pH 5.7, were used as starting material. The leaf disc transformation was essentially done as described by Deblaere *et al.* (1987). Midrib and edges were removed from the leaves and segments of ± 0.25 cm^2 were placed in the infection medium (B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES, pH 5.5 and 3% sucrose). To 10 ml of infection medium (in a 9 cm Petri dish) 25 µl of a late log culture of the *Agrobacterium* strain, grown in Min A, was added. After 2 days the leaf discs were washed with the infection medium supplemented with 500 mg/l cefotaxime. The leaf discs were placed on shoot-inducing medium [B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.7, 2% glucose, 40 mg/l adenine, 0.8% agar, 1 mg/l BAP, 0.1 mg/l indole acetic acid (IAA) and 500 mg/l cefotaxime] containing the selective agent (PPT or kanamycin). After 3–4 weeks the regenerating calli were transferred to the same medium without IAA and containing 200 mg/cefotaxine. Two to three weeks later, shoots were isolated and transferred to rooting medium (half the concentration of MS salts supplemented with 3% sucrose, 0.5 g/l MES pH 5.7 and 100 mg/l cefotaxime). Roots formed after 1–2 weeks. The rooted shoots were propagated as sterile shoot cultures or transferred to the greenhouse.

Tomato. Sterile shoot cultures of *L. esculentum* cv. *Lukullus*, were grown as described for tobacco. The midrib was removed from the leaves which were cut without wounding the segments of 0.25–1 cm^2. The segments were placed in the infection medium (B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.5, 3% sucrose, 40 mg/l adenine, 40 g/l mannitol, 0.5 mg/l transzeatine and 0.01 mg/l IAA). To 10 ml of infection medium (in a 9 cm Petri dish) 20 µl of a late log culture of the *Agrobacterium* strain, grown in Min A, was added. After 2 days the leaf discs were washed with the infection medium supplemented with 500 mg/l cefotaxime. The leaf discs were placed on shoot-inducing medium [B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.7, 0.5 g/l polyvinylpyrrolidone (PVP) 200 mg/l glutamine, 2% glucose, 40 mg/l adenine, 40 g/l mannitol, 0.6% agarose, 0.3 mg/l transzeatine, 0.01 mg/l IAA and 500 mg/l cefotaxime] containing 50–100 mg/l kanamycin. Each 5 days the osmotic pressure of the medium was lowered by decreasing the mannitol concentration with 10 g/l. After 1 month calli and meristems were separated from the leaf discs and placed on shoot-inducing medium without mannitol and IAA and containing 1 mg/l transzeatine and 200 mg/l cefotaxime. After small shoots had emerged, the calli were transferred to elongation medium (B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.7, 0.5 g/l PVP, 2% glucose, 40 mg/l adenine, 0.6% agarose, 1 mg/l transzeatine and 0.01 mg/l GA_3). The elongated shoots were transferred to medium containing half the concentration of MS salts supplemented with 2% sucrose, 0.5 g/l MES pH 5.7, 100 mg/l cefotamine and 0.7% agar. The rooted shoots were propagated as sterile shoot cultures or transferred to the greenhouse.

Potato. Transgenic potato were also obtained by a leaf disc infection method similar to the one for tobacco. The details of this procedure will be published elsewhere (De Block *et al.*, in preparation).

Herbicide applications

Plants were sprayed with a 2% aqueous solution of the formulated Basta® containing 20% D,L-PPT (Hoechst AG) or Herbiace® containing 33% bialaphos (Meiji Seika, Ltd) from the four sides in a 1 m^2 surface using air-brush line of Badger. Six week old F1 seedlings, planted separately in multijar plates, were sprayed with 8 l Basta/ha.

PAT assays

T.l.c. method. To 100 mg of tissue, 50–100 µl of extraction buffer [50 mM Tris-HCl, pH 7.5, 2 mM Na_2-EDTA, 0.15 mg/ml leupeptine, 0.15 mg/ml phenylmethylsulphonyl fluoride (PMSF), 0.3 mg/ml bovine serum albumin (BSA), 0.3 mg/ml DDT] was added with 5 mg PVP and sea sand. The tissue was ground with a glass rod in a punctured Eppendorf tube that contained a hydrophobic plug at the bottom. The Eppendorf tube was placed into a larger tube and centrifuged for 1 min at 1000 r.p.m. The extract which was recovered was clarified in an Eppendorf centrifuge for 5 min. To a diluted extract (12.5 µl) PPT (0.75 µl of a 1 mM stock in 50 mM Tris pH 7.5, 2 mM Na_2-EDTA) and [^{14}C]AcCoA (1.25 µl) (58.1 mCi/mmol; NEN) were added. The reaction mixture was incubated at 37°C for 30 min and spotted (6 µl) on a silicagel t.l.c. plate. Ascending chromatography was carried out in a 3 to 2 mixture of 1-propanol and NH_4OH (25% NH_3). ^{14}C was visualized by autoradiography (XAR-5-Kodak film overnight).

Spectrophotometric assay. Plant tissue (250 mg) was ground in 500 µl of the half concentrated extraction buffer without DDT. The extract was enriched for the PAT enzyme by a differential $(NH_4)_2SO_4$ precipitation (30–60%). The protein pellet was dissolved in 200 µl of buffer (50 mM Tris-HCl pH 7.5, 2 mM Na_2-EDTA). The protein concentration was measured relative to BSA as standard using the Biorad kit. The rest of the procedure was as described for chloramphenicol acetyl transferase (Shaw, 1975). The rate of PPT acetylation was quantified by measuring the free CoA sulfhydryl group coincident with transfer of the acetyl group to PPT. The reaction of the reduced CoA with 5,5′ dithiobis-2-nitrobenzoic acid (DTNB) yields a molar equivalent of free 5-thio-2-nitrobenzoic with a molar extinction coefficient of 13 600 at 412 nm. The net change in extinction per minute was divided by 13.6 to give the result in µmol/min of PPT-dependent DTNB generated. One unit of PAT is defined as 1 µmol of PPT acetylated per min at 37°C.

Isolation of plant RNA and hybridization

Leaf RNA was isolated essentially as described by Jones *et al.* (1985). A

Corteva Exhibit 2059 - Page 10 of 11
Inari v. Corteva - PGR2023-00022

*Bgl*II–*Stu*I fragment from pGSFR280, carrying the *bar* gene, was cloned into the SP6 vector pGEM-2 (Promega-Biotech). SP6 RNA polymerase was used to transcribe a radioactive RNA probe complementary to the *bar* mRNA. Northern hybridization experiments were as described by Jones *et al.* (1985).

Western blot

Aliquots of leaf extracts corresponding to 75 μg total extracted protein were electrophoresed on 12.5% SDS–polyacrylamide gel and transferred to a nitrocellulose filter. The filter was treated first with rabbit antibody to PAT, then with alkaline phosphatase-labelled goat anti-rabbit Ig. BC18/NBT alkaline phosphatase color development solution (Biorad) was used as substrate.

Ammonia determination in plant extracts

Leaves (250 mg) were extracted as described previously in 0.5 ml extraction buffer containing 0.1 g PVP. Insoluble material was pelleted and 1.6 ml trichloroacetic acid (0.3 M) was added to 0.4 ml supernatant. After 30 min the precipitate was removed by centrifugation. The supernatant was neutralized with KOH (10 mol/l), filtered and used for the determination of NH_4^+ (0.1–0.5 ml). In the presence of glutamate dehydrogenase and reduced nicotinamide-adenine nucleotide (NADH) ammonia reacts with α-ketoglutarate to L-glutamate, and NADH and ammonia are consumed stoichiometrically with the amount of ammonia. The assay was performed in 1 ml cuvettes containing 0.15 M triethanolamine pH 8.6, 11.1 mM α-ketoglutarate, 0.2 mM NADH and 8 U glutamate dehydrogenase. Consumption of NADH was monitored by recording the change in absorbance at 340 nm.

Acknowledgements

We thank Karin Tenning for preparation of the manuscript, Karel Spruyt and Stefaan Van Gysegem for making the illustrations. We thank Dr G.Donn for samples of phosphinothricin. Dr M.Lauwereys is acknowledged for purification and preparation of antibodies and Dr E.Krebbers for critical reading of the manuscript.

References

Botterman,J. (1986) PhD. Dissertation, State University of Ghent.
Bayer,E., Gugel,K.H., Hagele,K., Hagemaier,H., Jessipow,S., Konig,W.A. and Zahner,H. (1972) *Helv. Chim. Acta*, **55**, 224–239.
Comai,L., Facciotti,D., Hiatt,W.R., Thompson,G., Rose,R.E. and Stalker,D.M. (1985) *Nature*, **317**, 741–744.
Chaleff,R.S. and Ray,T.B. (1984) *Science*, **223**, 1148–1151.
Das Sarma,S., Tischer,E. and Goodman,H.M. (1986) *Science*, **232**, 1242–1244.
Deblaere,R., Bytebier,B., De Greve,H., Deboeck,F., Schell,J., Van Montagu,M. and Leemans,J. (1985) *Nucleic Acids Res.*, **13**, 4777–4788.
Deblaere,R., Reynaerts,A., Höfte,H., Hernalsteens,J.-P., Leemans,J. and Van Montagu,M. (1987) *Methods Enzymol.*, **153**, in press.
Donn,G., Tischer,E., Smith,J.A. and Goodman,H.M. (1984) *J. Mol. Appl. Genet.*, **2**, 621–635.
Ericson,M.C. (1985) *Plant Physiol.*, **79**, 923–927.
Gamborg,C.S. (1968) *Exp. Cell Res.*, **50**, 151–158.
Gebhardt,C., Oliver,J.E., Forde,B.G., Saarelainen,R. and Miflin,B.J. (1986) *EMBO J.*, **5**, 1429–1435.
Horsch,R.B., Fry,J.E., Hoffmann,N.L., Eichholtz,D., Rogers,S.G. and Fraley,R.T. (1985) *Science*, **227**, 1229–1231.
Jones,J.D.G., Dunsmuir,P. and Bedbrook,J. (1985) *EMBO J.*, **4**, 2411–2418.
La Rossa,R.A. and Falco,S.C. (1984) *Trends Biotechnol.*, **2**, 158–161.
Loyola-Vargas,V.M. and Sanchez De Jimenez,E.S. (1984) *Plant Physiol.*, **76**, 536–540.
Maliga,P., Breznovitz,A. and Marton,L. (1973) *Nature*, **244**, 29–30.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY.
Marton,L., Wullems,G.J., Molendijk,L. and Schilperoort, R.A. (1979) *Nature*, **277**, 129–130.
Miflin,B.J. and Lea,P.J. (1977) *Annu. Rev. Plant Physiol.*, **28**, 299–329.
Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, NY.
Murakami,T., Anzai,H., Imai,S., Satoh,A., Nagaoka,K. and Thompson,C.J. (1986) *Mol. Gen. Genet.*, **205**, 42–50.
Murashige,T. and Skoog,F. (1962) *Physiol Plant.*, **15**, 473–497.
Nagy,J.I. and Maliga,P. (1976) *Z. Pflanzenphysiol.*, **78**, 453–455.
Ogawa,Y., Tsuruoka,T., Inouye,S. and Niida,T. (1973) *Sci. Rep. Meiji Seika*, **13**, 42–48.
Sanders,P.R., Winter,J.A., Barnason,A.R., Rogers,S.G. and Fraley,R.T. (1987) *Nucleic Acids Res.*, **15**, 1543–1558.
Shah,D., Horsch,R., Klee,H., Kishore,G., Winter,J., Turner,N., Hironaka,C., Sanders,P., Gasser,C., Aykent,S., Siegel,N., Rogers,S. and Fraley,R. (1986) *Science*, **233**, 478–481.
Shaner,D.L. and Anderson,P.C. (1985) In *Biotechnology in Plant Science. Relevance to Agriculture in the Eighties*. Zaitlin,M., Day,P. and Hollaender,A. (eds), Academic Press, Inc., NY, p. 287.
Shaw,W.V. (1975) *Methods Enzymol.*, **43**, 737–755.
Shimabukuro,R.H., Frear,D.S., Swanson,H.R. and Walsh,W.C. (1971) *Plant Physiol.*, **47**, 10–14.
Skokut,T.A., Wolk,C.P., Thomas,J., Meeks,J.C. and Shaffer,P.W. (1978) *Plant Physiol.*, **62**, 299–304.
Tachibana,K., Watanabe,T., Sekizuwa,Y. and Takematsu,T. (1986) *J. Pesticide Sci.*, **11**, 33–37.
Velten,J. and Schell,J. (1985) *Nucleic Acids Res.*, **13**, 6981–6998.

Received on April 27, 1987; revised on May 26, 1987

Corteva Exhibit 2059 - Page 11 of 11
Inari v. Corteva - PGR2023-00022