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# *parB*: An auxin-regulated gene encoding glutathione *S*-transferase

(cell cycle/dedifferentiation/*Nicotiana tabacum*/protoplast)

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**ABSTRACT** **We have isolated an auxin-regulated cDNA, *parB*, from the early stage of cultured tobacco mesophyll protoplasts. The expression of *parB* was observed during transition from $G_0$ to the S phase of tobacco mesophyll protoplasts cultured *in vitro*. The predicted amino acid sequence of *parB* cDNA has 213 amino acid residues with a relative molecular weight of 23,965. Nucleotide sequence analysis revealed that *parB* cDNA has homology to glutathione *S*-transferase (GST; RX:glutathione R-transferase, EC 2.5.1.18) from several sources including plant and animal cells. When we introduced expression vector pKK233-2, which retains *parB* cDNA, into *Escherichia coli*, we could detect GST activity in the *parB* gene product. Accordingly a significant increase of GST activity was detected in the tobacco mesophyll protoplasts cultured in the presence of 2,4-dichlorophenoxyacetic acid. This is an example in which the function of auxin-regulated gene product is shown to be ascribed to a specific enzymatic activity. As GST, and its substrate glutathione, are shown to be related to cell proliferation as well as detoxification of xenobiotics in plant and animal cells, the role of *parB* is discussed in relation to the induction of proliferative activity in differentiated and nondividing mesophyll protoplasts of tobacco.**

Since the discovery of auxin as a plant hormone in 1934, auxins have been shown to regulate various aspects of plant growth and development. There have been numerous reports of physiological studies of auxin (1); however, the definite molecular mechanism of auxin action remains to be described. One way to elucidate the mode of action of auxin is to clone auxin-regulated genes and identify the function of their products. The study of hormonal activation of the transcription of these genes should resolve the signal transduction system involved in this process. Although several genes whose expression is regulated by auxin have been isolated, the function of the gene products has yet to be determined. In elongating sections of soybean hypocotyls and pea epicotyls, the application of auxin induces several genes whose function as well as regulatory sequences have yet to be determined (2–4). On the other hand, in a previous study we isolated from tobacco mesophyll protoplasts an auxin-regulated gene (*par*) that is expressed in response to exogenous auxins before the start of DNA synthesis (5). Subsequently, we have identified the auxin-responsive region in the 5′ flanking sequences of the *par* gene in a transient assay (6). However, the function of the *par* gene product has not yet been decisively determined. Thus, we intended to identify the function of gene products of auxin-regulated genes in order to understand the molecular mechanism of an auxin-mediated signal transduction system.

In this report, we describe the isolation of an auxin-regulated cDNA, *parB*, from tobacco mesophyll protoplasts cultured *in vitro*. *parB* has homology to glutathione *S*-transferase (GST; RX:glutathione R-transferase, EC 2.5.1.18) from several sources. As the gene product of the *parB* produced in *Escherichia coli* showed GST activity, the significance of GST as the gene product of an auxin-regulated *parB* cDNA is discussed.†

## MATERIALS AND METHODS

**Northern Blotting.** Protoplasts were prepared from mesophyll tissues of tobacco (*Nicotiana tabacum* L. cv. Xanthi nc) and RNA was isolated from the cultured protoplasts as described (5). As a Northern blotting probe, we used a 660-base-pair (bp) *Eco*RI/*Eco*RI fragment that covers the entire coding region of *parB* cDNA after labeling by random priming. Hybridization was carried out in 6× standard saline citrate (SSC)/1% SDS at 65°C. The filter was washed in 2× SSC/0.1% SDS at 65°C and autoradiographed using an intensifying screen. A blot was also probed with λA-1, a gene whose expression is not affected by 2,4-dichlorophenoxyacetic acid (2,4-D) (5).

**Expression of *parB* cDNA in *E. coli*.** The 660-bp *Eco*RI/*Eco*RI fragment covering the entire coding region of *parB*, whose ends had been rendered blunt, was introduced into the filled-in *Nco* I site of the expression vector pKK233-2 (7). The *Eco*RI site of the 5′ end of *parB* cDNA was derived from an *Eco*RI linker. The resultant plasmid was named pTRC-parB. Subsequently, *E. coli* JM109 transformed with pTRC-parB was induced to express parB protein by the addition of isopropyl β-D-thiogalactopyranoside to 1 mM and cultured for 4 hr. The collected cells were lysed and analyzed by SDS/PAGE using a 12% gel.

**Enzyme Assay of GST.** The enzyme assay was carried out according to standard procedures (8). In brief, the *E. coli* JM109 transformed with either pKK233-2 or pTRCparB was cultured for 4 hr and collected in a tube (1.5 ml) by centrifugation. The *E. coli* cells were suspended in 0.5 ml of 100 mM Tris·HCl, pH 7.5/1 mM dithiothreitol and lysed by sonication. After cell debris was removed by centrifugation, the enzyme activity of the supernatant was measured. To 10 μl of the supernatant was added 50 μl of 20 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 50 μl of 20 mM glutathione and 890 μl of 100 mM sodium phosphate buffer (pH 6.5). The enzyme reaction was carried out at 30°C for 30 min and enzyme activity was determined spectrophotometrically with the increase of absorbance at 340 nm.

## RESULTS

**Isolation of *parB* cDNA.** Although we reported previously that we isolated an auxin-regulated *par* gene from the cultured tobacco mesophyll protoplasts, two-dimensional gel electrophoresis by Meyer *et al.* (9) showed that auxin induces the appearance of two proteins that were not observed in the

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; 2,4-D, 2,4-dichlorophenoxyacetic acid; GST, glutathione *S*-transferase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. D90500).

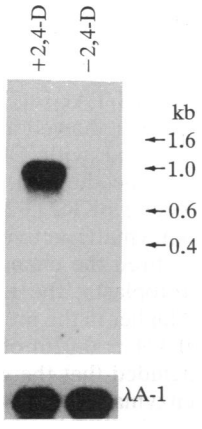


FIG. 1. Northern hybridization analysis of *parB* gene expression in tobacco mesophyll protoplasts. Tobacco mesophyll protoplasts were cultured for 24 hr in Nagata–Takebe (10) medium supplemented with 2,4-D ($4.5 \times 10^{-6}$ M) (+2,4-D) or without 2,4-D (−2,4-D). Each lane received 1 $\mu$g of poly(A)$^+$ RNA. kb, Kilobases.

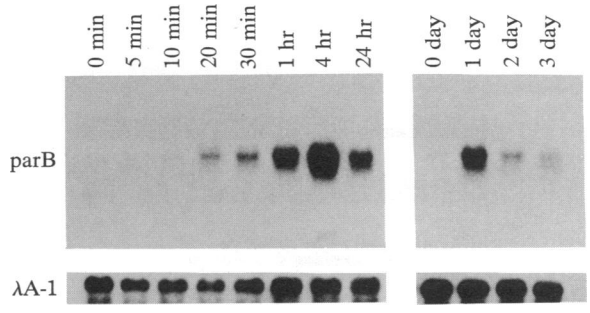


FIG. 2. Induction kinetics of *parB* mRNA by 2,4-D. 2,4-D ($4.5 \times 10^{-6}$ M) was added to the protoplasts, which had been precultured in Nagata–Takebe medium (10) without 2,4-D for 24 hr. RNA was extracted at the indicated times after addition of 2,4-D. Each lane received 20 $\mu$g of total RNA.

freshly prepared tobacco mesophyll protoplasts. Thus, we tried further to search for cDNA clones of other auxin-regulated genes in the cDNA library prepared from the cultured protoplasts essentially according to the procedure of Takahashi *et al.* (5). Then we identified a cDNA clone that was not hybridized to a full-length *par* cDNA clone (5), which we have named *parB* (protoplast auxin regulated). Accordingly, to avoid confusion (5), we have renamed the *par* gene *parA*.

**Expression of *parB* cDNA.** Northern hybridization revealed that *parB* was expressed in tobacco mesophyll protoplasts cultured for 24 hr in Nagata–Takebe medium (10) containing 2,4-D, but it was not expressed in the absence of 2,4-D (Fig. 1). The size of *parB* mRNA was ≈1000 nucleotides. Other auxins such as indole-3-acetic acid and 1-naphthaleneacetic acid induced *parB* as well (data not shown). However, other plant hormones such as cytokinin, gibberellic acid, and ethylene did not induce *parB* in place of auxin, and neither did stresses such as heat shock (data not shown).

The accumulation of *parB* mRNA was detected as early as 20 min and reached a maximum 4 hr after the addition of 2,4-D (Fig. 2). As active DNA synthesis has been observed from 24 to 48 hr in the cultured tobacco mesophyll protoplasts by incorporation of [$^3$H]thymidine (11), the time course experiment of the accumulation of *parB* mRNA shows that active expression of *parB* can be specified before the S phase and very probably during the transition from $G_0$ to $G_1$. After 48 hr, when active cell division was observed, *parB* accumulation was almost suppressed. This time course was very similar to that of *parA* gene expression (5).

**DNA Sequence of *parB* cDNA and Its Homology to GST.** The sequence of *parB* cDNA was determined and is presented in Fig. 3, along with the derived amino acid sequence. The longest open reading frame starting with methionine is 639 bp, corresponding to 213 amino acid residues with a relative molecular weight of 23,965. In the 3′ untranslated region, the *parB* cDNA clones are polyadenylylated at different positions and contain two canonical polyadenylylation signals, AATAAA and ATTAAA. The ATTTA sequence, which was observed in *parA* mRNA, is present within the 3′ untranslated region. This sequence could confer the instability on *parB* mRNA, as it is reported to play a role in the posttranslational regulation of gene expression by conferring instability on mRNAs of various mammalian genes (5). When the derived amino acid sequence was scanned against the National Biomedical Research Foundation protein data base for

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1          AGCTGTATATCCAGAAATT ATG GCG ATC AAA GTC CAT GGT AGC CCC ATG TCA ACT GCA ACC ATG AGA GTT    70
1                              Met Ala Ile Lys Val His Gly Ser Pro Met Ser Thr Ala Thr Met Arg Val    17

71   GCT GCT TGC CTC ATC GAG AAG GAG CTG GAT TTT GAG TTT GTC CCT GTT GAT ATG GCC TCT GGC GAA CAC AAG   142
18   Ala Ala Cys Leu Ile Glu Lys Glu Leu Asp Phe Glu Phe Val Pro Val Asp Met Ala Ser Gly Glu His Lys    41

143  AAG CAC CCT TAC CTT TCC CTC AAT CCT TTT GGT CAA GTA CCA GCA TTT GAA GAT GGG GAC TTG AAG CTT TTT   214
42   Lys His Pro Tyr Leu Ser Leu Asn Pro Phe Gly Gln Val Pro Ala Phe Glu Asp Gly Asp Leu Lys Leu Phe    65

215  GAA TCA AGG GCA ATC ACC CAA TAC ATT GCT CAT GTT TAT GCT GAC AAT GGC TAT CAA CTA ATA CTC CAA GAT   286
66   Glu Ser Arg Ala Ile Thr Gln Tyr Ile Ala His Val Tyr Ala Asp Asn Gly Tyr Gln Leu Ile Leu Gln Asp    89

287  CCA AAG AAG ATG CCC AGT ATG TCA GTA TGG ATG GAA GTA GAA GGC CAA AAA TTT GAA CCC CCG GCT ACA AAA   358
90   Pro Lys Lys Met Pro Ser Met Ser Val Trp Met Glu Val Glu Gly Gln Lys Phe Glu Pro Pro Ala Thr Lys   113

359  TTA ACA TGG GAA CTA GGC ATA AAA CCA ATT ATC GGC ATG ACC ACA GAT GAT GCT GCT GTG AAG GAA AGC GAA   430
114  Leu Thr Trp Glu Leu Gly Ile Lys Pro Ile Ile Gly Met Thr Thr Asp Asp Ala Ala Val Lys Glu Ser Glu   137

431  GCG CAA TTG TCT AAG GTT CTT GAC ATA TAC GAA ACT CAG TTG GCA GAG TCA AAA TAC TTA GGT GGA GAC TCT   502
138  Ala Gln Leu Ser Lys Val Leu Asp Ile Tyr Glu Thr Gln Leu Ala Glu Ser Lys Tyr Leu Gly Gly Asp Ser   161

503  TTT ACA CTG GTT GAT TTG CAC CAC ATC CCA AAT ATA TAT TAC TTG ATG AGT TCA AAA GTT AAG GAA GTG TTT   574
162  Phe Thr Leu Val Asp Leu His His Ile Pro Asn Ile Tyr Tyr Leu Met Ser Ser Lys Val Lys Glu Val Phe   185

575  GAT TCG CGC CCT CGT GTG AGT GCA TGG TGT GCT GAT ATT TTG GCC AGG CCA GCT TGG GTT AAG GGA TTG GAG   646
186  Asp Ser Arg Pro Arg Val Ser Ala Trp Cys Ala Asp Ile leu Ala Arg Pro Ala Trp Val Lys Gly Leu Glu   209

647  AAG CTG CAA AAA TAAAGAATTCATGAGCTAATGGATGAGCATATTCCCAGAAGCCTAATTTCTGTTTCATCTCTTGTGTTTTCTGTCTGAA   737
210  Lys Leu Gln Lys                                                                                 213
                                                                                    ↓
738  GTAGTTGTCTTGCAATACAATAAAACAGTCATAAATATCTATGTCAAGAGATATAATCATGTGGTGTTTGTAGACTCAGTCTCATCTCCTTTCCA   834

835  TTTCCTATTAAATTGAACCGTTTGGTTTATCAAATTCTAAATATCTTATTTAATAAGCATATAAAATATAATTATATGGTAAAAAAAAAAAAAA   929
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FIG. 3. Nucleotide sequence of *parB* cDNA and deduced primary structure of the protein. The DNA sequence of the longest open reading frame of *parB* is shown. The alternative 3′ end of *parB* cDNA is marked by an arrow. Two potential polyadenylylation signal sequences are underlined. An ATTTA sequence, which possibly confers mRNA instability (5), is boxed.


FIG. 4. Amino acid sequence of *parB* cDNA is compared with those of maize GST III (MGST III) (15), rat placental GST-P (Rat GST) (16), human GST 2 (Human GST) (17), and rat lysophospholipase (Rat LPL) (18). Identical amino acids are shaded. Positions of homologous regions of *parB*, rat GST, human GST, and rat LPL are indicated in parentheses with amino acid residue numbers.

related sequences, significant homology was found between the parB and maize GST. GSTs represent a family of enzymes that catalyze the conjugation of glutathione to a variety of electrophiles (12, 13). This family of enzymes is widely distributed in mammals, insects, and higher plants. In human beings, at least seven different GSTs have been identified (14). Most of these enzymes have distinct but overlapping specificities for substrates. Among the three isozymes of GST thus far identified in maize (15), the parB was most homologous to maize GST III and the extent of homology between these two proteins was 46% (Fig. 4), whereas the comparison of *parB* to maize GST I revealed 38% identity. The homology to mammalian GSTs (16, 17) was limited to the region of amino acid sequence between residues 52 and 75 (Fig. 4), which suggests that this region could be the functional domain for enzymatic activity of GST. It was also found that this region has a homology to rat lysophospholipase (18) (Fig. 4), although the significance of this observation awaits further investigation.

**Gene Product of *parB* cDNA.** To show conclusively GST activity of the *parB* gene product, we transformed *E. coli* with an expression vector pKK233-2 (7), into which was inserted the *parB* cDNA placed downstream to a *trc* promoter (Fig. 5*A*). In this construct, eight extra amino acids were fused to the N terminus of the parB protein. When the *E. coli* was induced by isopropyl β-D-thiogalactopyranoside, the recombinant plasmid pTRCparB expressed *parB* and produced a protein whose molecular weight corresponded to the predicted parB product on SDS/PAGE (Fig. 5*B*). The resultant sonicated extracts of *E. coli* showed a conspicuous level of GST activity when assayed with CDNB as a substrate according to a standard procedure (8), whereas *E. coli* cells transformed with the vector pKK233-2 alone showed only a background level of enzymatic activity (Fig. 5*C*). Subsequently, when we measured the change of GST activity in tobacco mesophyll protoplasts, the level of GST activity became conspicuously higher in the presence (0.523 ± 0.039) than in the absence (0.374 ± 0.026) of 2,4-D after 24 hr of culture. Thus, we concluded that the gene product of *parB* cDNA contains GST enzymatic activity and auxin treatment brings about the increase in GST level in tobacco mesophyll protoplasts.

## DISCUSSION

In this paper, we describe isolation of an auxin-regulated cDNA, *parB*, from tobacco mesophyll protoplasts cultured *in vitro*. Since the predicted gene product of *parB* cDNA showed homology to GSTs from various sources, we examined whether the *parB* gene products expressed in *E. coli* had GST activity. We could unambiguously detect the GST activity in the gene product of *parB* cDNA. Furthermore, we have detected the increase of GST activity due to auxin in the cultured tobacco mesophyll protoplasts. As this is the first report in which the gene product of an auxin-regulated gene is ascribed to a specific enzymatic activity, the next intriguing question should be how GST as the parB product is involved in the induction of meristematic activity of differentiated and nondividing tobacco mesophyll protoplasts.

GSTs belong to a category of enzymes that catalyze the conjugation of a variety of electrophilic xenobiotics with glutathione, resulting in detoxification of animal and plant cells (12, 13). On the other hand, it has been suggested recently that specific expression of placental GSTs would be closely related to the process of neoplastic transformation (19). In fact, a placental GST has been shown to be induced specifically at an early stage of chemical hepatocarcinogenesis in rat and its causal relationship is almost 100% (20). Moreover, it has been reported that experimentally induced expression of the activated *ras* gene results in concomitant expression of a placental GST in cultured rat liver cells in

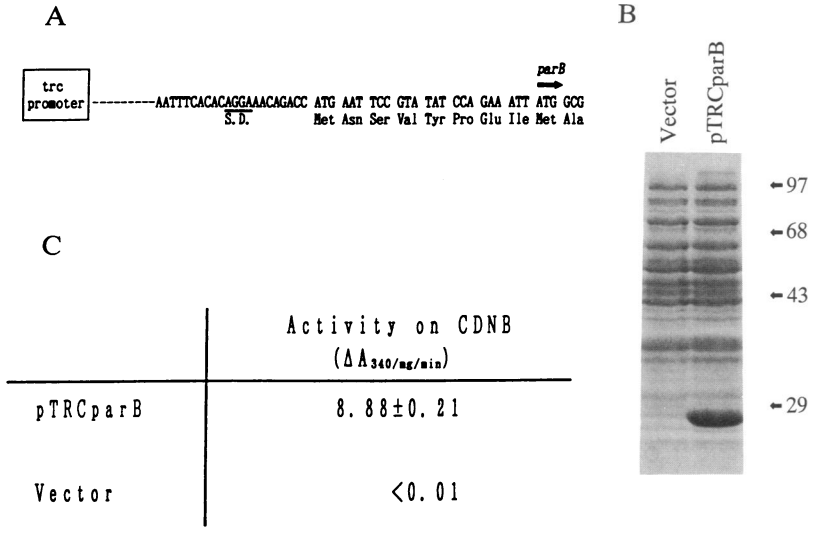


FIG. 5. Expression of *parB* cDNA in *E. coli*. (*A*) Structure of pTRCparB overproducing parB protein. (*B*) SDS/PAGE of the parB proteins overproduced in *E. coli*. Numbers on right are kDa. (*C*) GST assay using CDNB as the substrate. SDs are calculated from five experiments.

association with carcinogenesis (21). The involvement of glutathione, a substrate for GST, has been implicated in cell proliferation, as Suthanthiran *et al.* (22) showed that glutathione regulates activation-dependent DNA synthesis in the T lymphocytes stimulated with antigens. Thus, two possibilities for the function of GST as the gene product of *parB* can be supposed. One is that exogenous auxins as xenobiotics could be detoxified by GST, although thus far detoxification of auxins by GSTs has not been known (1). The other possibility is that the expression of *parB* could be related to the proliferative activity of tobacco mesophyll protoplasts. In either possibility, it should be essential to search substrates for the GST as a gene product of *parB* in tobacco mesophyll protoplasts.

Finally, it should be noted that elucidation of the intermediate signal transduction system of *parB* gene expression should be an intriguing question for further study, as the link between auxin application and enzymatic activity of GST as a product of the *parB* gene has been clearly proven.

**Note Added in Proof.** A detailed kinetics of *parA* gene expression will be published by Takahashi *et al.* (23).

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1. Thimann, K. V. (1972) in *Plant Physiology: A Treatise, VIB Physiology of Development: The Hormones*, ed. Steward, F. C. (Academic, New York), pp. 3–365.
2. Key, J. L., Kroner, P., Walker, J., Hong, J. C., Ulrich, T. H., Ainley, W. M., Gantt, J. S. & Nagao, R. T. (1986) *Philos. Trans. R. Soc. London Ser. B* **314,** 427–440.
3. Guilfoyle, T. J. (1986) *CRC Crit. Rev. Plant Sci.* **4,** 247–276.
4. Theologis, A. (1986) *Annu. Rev. Plant Physiol.* **37,** 407–438.
5. Takahashi, Y., Kuroda, S., Tanaka, T., Machida, Y., Takebe, I. & Nagata, T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9279–9283.
6. Takahashi, Y., Niwa, Y., Machida, Y. & Nagata, T. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8013–8016.
7. Amann, E. & Broslus, J. (1985) *Gene* **40,** 183–190.
8. Mannervik, B. & Guthenberg, C. (1981) *Methods Enzymol.* **77,** 231–235.
9. Meyer, Y., Aspart, L. & Chatier, Y. (1984) *Plant Physiol.* **75,** 1027–1033.
10. Nagata, T. & Takebe, I. (1970) *Planta* **92,** 301–308.
11. Zelcer, A. & Galun, E. (1976) *Plant Sci. Lett.* **7,** 331–336.
12. Rushmore, T. H., King, R. G., Paulson, K. E. & Pickett, C. B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3826–3830.
13. Chasseud, L. F. (1979) *Adv. Cancer Res.* **29,** 175–273.
14. Rushmore, T. H., King, R. G., Paulson, K. E. & Pickett, C. B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3826–3830.
15. Grove, G., Zarlengo, R. P., Timmerman, K. P., Li, N.-P., Tam, M. F. & Tu, C.-P. (1988) *Nucleic Acids Res.* **16,** 425–438.
16. Suguoka, Y., Kano, T., Okuda, A., Sakai, M., Kitagawa, T. & Muramatsu, M. (1985) *Nucleic Acids Res.* **13,** 6049–6059.
17. Board, P. G. & Webb, G. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2377–2381.
18. Han, J. H., Stratowa, C. & Rutter, W. J. (1987) *Biochemistry* **77,** 231–235.
19. Sakai, M., Okuda, A. & Muramatsu, M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9456–9460.
20. Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. & Sato, K. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3964–3968.
21. Li, Y., Seyama, T., Godwin, A. K., Winokur, T. S., Lebovitz, R. M. & Lieberman, M. W. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 344–348.
22. Suthanthiran, M., Andersen, M. E., Sharma, V. K. & Meister, A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3343–3347.
23. Takahashi, Y., Kusaba, M., Hiraoka, Y. & Nagata, T. (1991) *The Plant J.* **1,** in press.