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

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Communicated by Georg Melchers, September 16, 1991 (received for review April 20, 1991)

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_2 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 definitive 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 hypocotyl and pea epicotyl, 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. Xanthic) and RNA was isolated from the cultured protoplasts as described (5). As a Northern blotting probe, we used a 660-base-pair (bp) EcoRI/EcoRI 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 AA-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 EcoRI/EcoRI 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 EcoRI site of the 5' end of parB cDNA was derived from an EcoRI 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 pTRC-parB 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

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).
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. 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 × 10−6 M) (+2,4-D) or without 2,4-D (−2,4-D). Each lane received 1 μg of poly(A) RNA. kb, Kilobases.](image)

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![Fig. 2. Induction kinetics of parB mRNA by 2,4-D. 2,4-D (4.5 × 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 μg of total RNA.](image)

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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 polyadenylation signal sequences are underlined. An ATTAA sequence, which possibly confers mRNA instability (5), is boxed.](image)

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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. 5A). 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. 5B). 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. 5C). 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 neoplastic 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

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**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).

We wish to thank Dr. Rick Walden (Cologne) for his critical reading of the manuscript. This study was supported in part by grants from the Ministry of Education, Culture and Science of Japan, from the Ministry of Agriculture, Forestry and Fisheries of Japan (to T.N.) and from the Agency of Science and Technologies of Japan (to Y.T.).


