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Location of the cis-acting auxin-responsive region in the promoter of the *par* gene from tobacco mesophyll protoplasts

(electroporation/β-glucuronidase/*Nicotiana tabacum*/promoter)

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ABSTRACT **We have isolated a genomic clone of an auxin-regulated *par* gene, which is expressed during the transition from G_0 phase to S phase in the early stage of tobacco mesophyll protoplasts cultured *in vitro*, from a tobacco genomic library using the par cDNA as a probe. When a chimeric gene, in which a reporter gene for bacterial β-glucuronidase (GUS) was placed downstream of the 5′ flanking sequences of the *par* gene, was introduced into tobacco mesophyll protoplasts by electroporation, the chimeric gene elicited auxin-regulated expression of GUS activity. Because deletion of a 111-base-pair (bp) direct repeat in the 5′ flanking sequences of the *par* gene abolished the auxin-induced GUS activity, it is deduced that in the 111-bp direct repeat of the *par* gene promoter is localized an auxin-responsive region, which regulates auxin-mediated activation of transcription.**

One of the fundamental questions in plant molecular biology concerns the mechanism of action of plant hormones, which regulate the fate of cells in tissues and organs during growth, development, and differentiation. Among these, the role of auxin, which was first discovered as a plant hormone more than 60 years ago, is of foremost importance, since it has been shown to exert drastic influences upon numerous aspects of plant growth and development (1). Despite a mass of literature on physiological studies of auxins, very little information is available on the action mechanism of auxin at the molecular level. Although several auxin-regulated genes have been isolated from elongating tissues of pea (2) and soybean (3, 4), regulatory sequences involved in hormonal activation of transcription of these genes have yet to be identified. On the other hand, it has been well-established from earlier studies of plant tissue culture that auxin is necessary for inducing cell division (5); in tobacco mesophyll protoplasts we have shown that the presence of auxin is an absolute requirement for the induction of cell division (6). From this material we recently have isolated the cDNA of a gene (*par*) that is abundantly expressed in response to auxins before the onset of cell division (7). The conspicuous accumulation of par mRNA before the initiation of DNA synthesis in tobacco mesophyll protoplasts suggests that the *par* gene product may play a pivotal role in the initiation of meristematic activity in nondividing somatic cells. To search for regions responsible for enhancing expression in response to auxin, we analyzed the promoter of the *par* gene, using β-glucuronidase (GUS) as a reporter gene, after delivery into tobacco mesophyll protoplasts by electroporation. In fact, we could localize the cis-acting auxin-responsive region in the promoter of the *par* gene.¶

MATERIALS AND METHODS

Construction of Chimeric Plasmids Harboring the *par* Gene Promoter. A 0.8-kilobase (kb) *Mae* II fragment of the *par* gene promoter was isolated from tobacco genomic library by using the *par* gene cDNA as a probe. This fragment, retaining the cap site and coding region for five amino acids including the initiation codon (whose ends were rendered blunt), was introduced into the filled-in *Bam*HI site of the plasmid pBI101 (8). In this construct, the *par* gene coding region for five amino acids and the gene for GUS were connected in frame. This chimeric *par*–GUS gene was cloned in pUC18, which was named pPARΔMGUS. pPARΔMGUS was digested with *Sal* I and *Pst* I, and treated successively with *Exo* III, mung bean nuclease, DNA polymerase I (Klenow fragment), phage T4 DNA ligase, and *Sal* I to generate a set of deletion mutants (pPARΔ41GUS, pPARΔ45GUS, pPARΔ106GUS, pPARΔ-108GUS, and pPARΔ2GUS). A 0.3-kb *Xba* I fragment was constructed in the same way as the *Mae* II fragment. The ends of these shortened fragments were identified by DNA sequence analysis.

Protoplast Isolation. Mesophyll protoplasts were isolated from leaves of tobacco (*Nicotiana tabacum* L. cv. Xanthi nc) according to the two-step procedure as described (7).

Electroporation. To remove sporadically observed *par* gene expression (7), the protoplasts were incubated in NT 70 medium (6) without 2,4-dichlorophenoxyacetic acid (2,4-D) for 24 hr under continuous illumination from fluorescent tubes [2000 luxes (lx)] at 25°C and were washed once with a buffer consisting of 0.6 M mannitol, 70 mM KCl, and 5 mM Mes (pH 5.8). They were then suspended in 1.5 ml of an electroporation buffer containing 0.6 M mannitol, 70 mM KCl, 5 mM Mes (pH 5.8), and 1% polyethylene glycol 1540 (Koch-Light Laboratories, Colnbrook, England) at a cell density of 2×10^5 per ml. After incubation of the protoplast suspension on ice for 30 min, plasmid was added to 6.7 μg/ml without carrier DNA. Electroporation was carried out as described by Nagata (9) except that a power supply of 200 V was applied. Each sample was then washed once with 0.6 M mannitol, divided into two equal aliquots, and suspended in 3 ml of NT 70 medium (6) with or without 4.5 μM 2,4-D and cultured under continuous illumination from fluorescent tubes (2000 lx) at 25°C for 40 hr. A recent experiment established that a 24-hr incubation is sufficient for the GUS assay.

GUS Assay. For the GUS assay, cultured protoplasts were collected by centrifugation at $100 \times g$ for 2 min and lysed by sonication with a sonicator (Tomy UR-20P) for 30 sec after the addition of 0.4 ml of a lysis buffer described by Jefferson (8). After the removal of any solid material from the extract by centrifugation at $14,000 \times g$ for 10 min, a portion of the supernatant was used to determine protein content. The

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Abbreviations: ABP, auxin-binding protein; 2,4-D, 2,4-dichlorophenoxyacetic acid; GUS, β-glucuronidase.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. D90215).

remainder of the supernatant was assayed for GUS activity by a fluorogenic method (8).

RESULTS

Isolation of the 5′ Flanking Region of the *par* Gene. A tobacco genomic clone bearing the *par* gene was isolated by screening a genomic library using the *par* gene cDNA as a probe. An *Eco*RI 2.3-kb fragment of the resultant genomic clone was anticipated to retain the promoter region of the *par* gene and this fragment was subcloned and its sequence determined (Fig. 1). Transcription was found to start with the adenine residue located 70 bp upstream from the first ATG, and the 5′ flanking region of the *par* gene carries a 111-bp direct repeat, 10 ATATAG repeats, and a TGA1a,b binding site, TGACG (10) (Fig. 1). The sequence of the transcribed region of the *par* gene was perfectly matched with the cDNA. A candidate of a TATA box is located between positions −53 and −57. A preliminary Southern blot analysis of genomic DNA revealed that *par* is a member of a small gene family (data not shown).

Location of the cis-Acting Auxin-Responsive Region in the *par* Gene Promoter. First, the responsiveness of the *par* promoter to auxin was examined. A chimeric gene was constructed in which a 0.8-kb *Mae* II fragment carrying the *par* promoter was fused to the coding region of the *Escherichia coli* GUS gene (GUS) (pPARΔMGUS, see Fig. 2). This chimeric gene was introduced by electroporation into tobacco mesophyll protoplasts that had been cultured in Nagata and Takebe medium (NT 70) (6) without 2,4-D for 24 hr. After electroporation, the protoplasts were cultured in NT 70 medium (6) either in the presence or absence of 4.5 μM 2,4-D for 40 hr. GUS activity was determined in each sample as described by Jefferson (8). In this context it is worth noting that subtraction of 2,4-D from the medium had little effect on the synthesis of RNA and protein (11). GUS expression increased drastically when the electroporated protoplasts were cultured in the presence of 2,4-D but was present only at a very low level when they were cultured in the absence of 2,4-D. This observation implies that the *par* gene is transcriptionally regulated and that the 5′ flanking region is capable of driving GUS gene expression in an auxin-responsive manner. For each electroporation, it was necessary to observe the frequency of damaged protoplasts upon electroporation under a microscope, as it was found that the increase of the percentage of damaged protoplasts was reciprocally proportional to the level of GUS activity enhanced by auxin. Only if the percentage of damaged protoplasts did not exceed 30%, the GUS assay was carried out. When the frequency of the damaged protoplasts exceeded this level, the increment of GUS activity enhanced by auxin became very small. Among other conditions, osmotic pressure of the electroporation medium was a critical one affecting the damage of protoplasts. In earlier experiments, 0.5 M mannitol was used as described in Okada *et al.* (12), but in some cases more than 50% of protoplasts were broken upon electroporation. Increase of osmotic pressure by increasing the mannitol to 0.6 M alleviated considerably this breakage of protoplasts.

To confine the auxin-responsive regions of this promoter, we constructed a set of deletion mutants by the use of *Exo* III as shown in Fig. 2 (pPARΔ41GUS, pPARΔ45GUS, pPARΔ106GUS, pPARΔ108GUS, and pPARΔ2GUS). In three independent experiments, one of which is exemplified in Fig. 3, pPARΔ106GUS, pPARΔ108GUS, and pPARΔ2GUS did not show any significant difference between the presence and absence of auxin during the culture of protoplasts, but the mutants (pPARΔ45GUS to pPARΔ41GUS reproducibly displayed a stepwise increase of GUS expression in response to auxin treatment, and pPARΔMGUS (0.8-kb *Mae* II fragment) showed maximum expression (Fig. 3). This level was 20-fold more than that of the cauliflower mosaic virus 35S promoter, which showed no significant response to auxin treatment (data not shown).

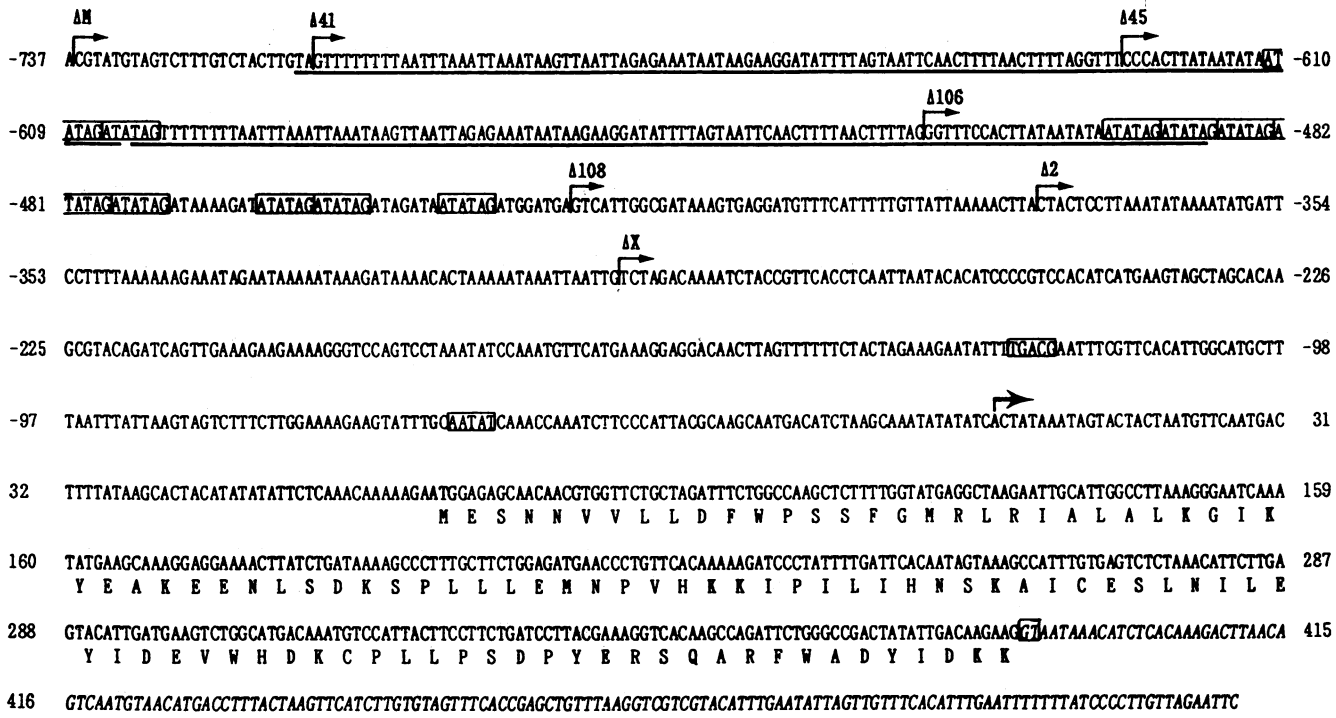

FIG. 1. Nucleotide sequence of the *par* gene promoter region. The transcription start site was determined by primer extension analysis (7) and is indicated by an arrow. The 111-bp direct repeat is underlined. The TATA box-like sequence, TGA1a,b binding sequence (10), and ATATAG repeats are boxed. The *Exo* III deletion end points are indicated. In this figure the first exon and a part of an intron of the *par* gene are included.

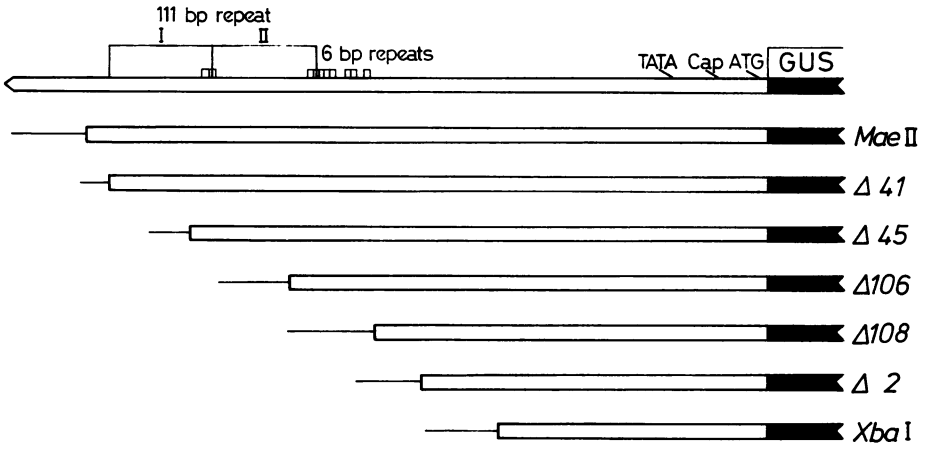

FIG. 2. Schematic presentation of chimeric plasmids of *par*–GUS gene constructs. pPARΔ41GUS, pPARΔ45GUS, pPARΔ106GUS, pPARΔ108GUS, pPARΔ2GUS, and pPARΔXGUS were produced after pPARΔMGUS was digested with *Sal* I and *Pst* I and treated successively with *Exo* III, mung bean nuclease, DNA polymerase I (Klenow fragment), T4 DNA ligase, and *Sal* I.

On the other hand, when pPARΔMGUS was introduced into protoplasts of the rapidly growing tobacco BY-2 cell line (13), the increase in GUS activity in response to auxin was only 1.5- to 2-fold (data not shown), in contrast to the 7- to 10-fold increase in GUS activity in the tobacco mesophyll protoplasts described above. Thus, the response of the *par* gene promoter to auxin may be dependent on cell and tissue type, and the *par* promoter responds to auxin preferentially in tobacco mesophyll protoplasts. This observation differs from that of another plant hormone, abscisic acid, in which a wheat promoter directs expression in response to abscisic acid in protoplasts from heterologous tissues of cultured rice cells (14).

DISCUSSION

In this paper we report that we have isolated the promoter of the *par* gene, which is expressed during the transition from G_0 to S phase of the cell cycle in the cultured tobacco mesophyll protoplasts, and we have demonstrated auxin responsiveness of this promoter when we delivered by electroporation chimeric plasmids of the GUS gene placed downstream of the *par* promoter into tobacco mesophyll protoplasts. This promoter directs a 20-fold increased expression of GUS gene compared with the cauliflower mosaic virus 35S promoter, and the presence of auxin caused a 7- to 10-fold enhancement. Thus, auxin responsiveness was clearly demonstrated in the promoter of auxin-responsive genes. However, since this auxin-responsive promoter showed some weak expression of GUS activity without auxin treatment (Fig. 3), some other auxiliary factors should be involved in the expression of the *par* gene promoter. Alternatively, a low level of auxin production could be restored during the culture of protoplasts.

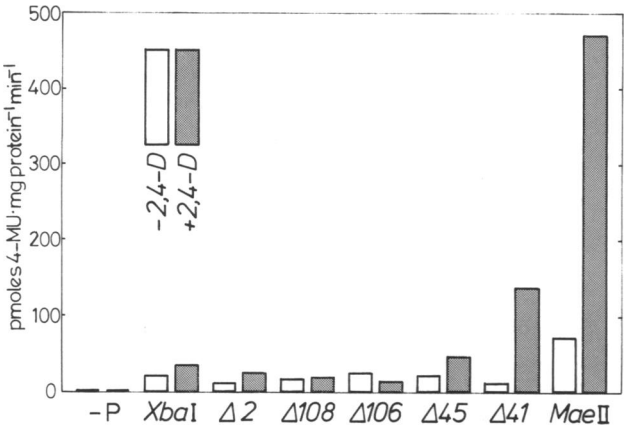

FIG. 3. GUS assay in electroporated tobacco mesophyll protoplasts. GUS assay of the electroporated protoplasts was carried out after a 40-hr incubation. Activity is expressed in pmol of 4-methylumbelliferone (4-MU) produced per mg of protein in the protoplast extract per min (8). The columns show GUS activity in cell extracts from protoplasts that were electroporated with the plasmid constructs shown in Fig. 2. Filled bars represent GUS activity from cell extracts from protoplasts cultured with 2,4-D; open bars represent protoplasts cultured without 2,4-D. The two columns at the extreme left (−P) show protoplasts that received the GUS gene lacking a promoter (pBI101).

Deletion mutants of the *par* promoter showed a stepwise increase of GUS activity when using pPARΔ45GUS to pPARΔ41GUS and the maximum expression with pPARΔMGUS. As there is a unique 111-base-pair (bp) direct repeat along with this region, in which pPARΔ41GUS covers 111 bp and pPARΔMGUS covers one complete 111-bp direct repeat (Fig. 1), a possible interpretation is that auxin-responsive elements could be included in the 111 bp, and the lower level of GUS activity by pPARΔ41GUS in comparison with pPARΔMGUS could be due to the 2-bp deletion at the left border of 111-bp direct repeat, although another possibility is that the presence of another auxin-responsive element in the 25-bp region between pPARΔ41GUS and pPARΔMGUS cannot be excluded. If the former were the case, sequences at the left border of this 111 bp should be responsive to auxin. Then the evidence that a sequence (AGTTTTTT) observed at the left border is also observed in the promoter regions of other auxin-regulated genes of *Gmhsp* 26-A (3) as well as *dbp* from dividing tissues of *Arabidopsis thaliana* (15) should not be a simple coincidence. The proof of this possibility awaits further examination. However, the sequences that are supposed to be auxin-responsive by Ainley *et al.* (16) and by An *et al.* (17) are not observed in the auxin-responsive region of the *par* promoter.

On the other hand, Barbier-Brygoo *et al.* (18) showed that the treatment of tobacco mesophyll protoplasts with antibodies against a maize auxin-binding protein (ABP) (19, 20) alleviates the shift of membrane potential induced by auxins and supposed that the physiological changes induced by auxin are mediated by the ABP of the protoplast surface. In fact, we have identified a 22-kDa ABP in the plasma membrane of tobacco mesophyll protoplasts that is immunologically identical to the maize ABP (S. Shimomura, W. Liu, N. Inohara, M. Futai, and T.N., unpublished data). In this context the relationship between ABP and *par* gene expres-

sion should also be interesting. Once trans-acting factors have been characterized further, it will be possible to describe a plant hormone signal transduction sequence from auxin supply to the expression of specific genes resulting in the passage of plant cells from one stage of the cell cycle to another. An answer to the molecular action of auxin in this system may then be available. It will also be of great interest to determine the action of auxin in elongating tissues with reference to the mechanisms that operate in dividing cells.

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