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Molecular mechanisms of herbicide-inducible gene expression of tobacco CYP71AH11 metabolizing the herbicide chlorotoluron

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ABSTRACT

Tobacco cytochrome P450 (CYP) 71AH11 metabolized the herbicide chlorotoluron, and its mRNA level was increased in tobacco culture cells by the treatment of 2,4-D. In order to clarify molecular mechanisms of induced gene expression of *CYP71AH11* by herbicide treatment, a 1574-bp 5′-flanking region of *CYP71AH11* was cloned, ligated to the reporter β-glucuronidase (GUS) gene, and then transformed into tobacco plants. The GUS activity in the transgenic tobacco plants was highly induced by bromoxynil treatment, followed by 2,4-D. Chlorotoluron was slightly increased the GUS activity. The bromoxynil-increased GUS activity was partially repressed by the antioxidants, suggesting that reactive oxygen species may be involved in activation of the 5′-flanking region of *CYP71AH11* by bromoxynil treatment. Deletion and mutation assays showed that the region CD (−1281 to −770 bp from the start codon of *CYP71AH11*) was important, but not sufficient, for response to bromoxynil. Electrophoretic mobility shift assays and southwestern blotting revealed that the sequences AAAAAG, and GAACAAAC and GAAAATTC in the CD region were important for interaction to the nuclear proteins of <30 and ≈75 kDa, respectively. Particularly, interaction between AAAAAG and <30 kDa proteins was increased by bromoxynil treatment. These results gave a cue for understanding the bromoxynil-induced gene expression of *CYP71AH11*, which may contribute to herbicide tolerance and selectivity in crop plants.

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1. Introduction

Cytochrome P450s (CYPs) compose a superfamily of hemoproteins which play important roles on oxidative metabolism of xenobiotics in mammals. Certain CYP isoforms, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, are involved in more than 90% of phase I of drug metabolism in human livers [1]. In higher plants, it was reported for the first time in 1968 that the herbicide monuron was metabolized in a microsomal fraction of cotton seedlings [2]. Then, CYP76B1 in Jerusalem artichoke was reported to involve in mono- and di-*N*-demethylation of the herbicides chlorotoluron and isoproturon [3]. Tobacco CYP81B2 and Jerusalem artichoke CYP73A1 catalyzed ring-methyl hydroxylation of chlorotoluron [4,5]. Soybean CYP71A10 functioned as an *N*-demethylase toward fluometuron, linuron, and diuron, and as a ring-methyl hydroxylase toward chlorotoluron [6].

The drug-metabolizing CYPs are often induced by xenobiotics in mammals, and nuclear receptors play an important role on induced gene expression of these *CYP* genes. Phenobarbital induced CYP subfamilies 2A, 2C, 3A, and especially 2B [7]. Phenobarbital also induced maize CYP72A5 and CYP92A1, wheat CYP709C1, and other isoforms in higher plants [8–10]. It was reported that aminopyrine induced CYP76B1 and CYP73A1 in Jerusalem artichoke [3,11]. The herbicide safener 1,8-naphthalic anhydride induced CYP81C1 and CYP81C2 in tobacco [5], and CYP71C1, CYP71C3, CYP72A5, CYP73A7, and CYP92A1 in maize [10]. Based on these reports, it was presumable that there are *cis*-elements for xenobiotic response in 5′-flanking regions of these CYP genes. However, neither nuclear receptors nor *cis*-elements participating in xenobiotic responsive expression of these *CYP* genes have been identified yet in higher plants.

It was reported that reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical, and hydroxyl radicals were accumulated in higher plants on exposure to the herbicide chemicals atrazine [12], acetochlor [13], dinoterb [14], 2-benzoxazolinone

Abbreviations: CYP, cytochrome P450; 2,4-D, (2,4-dichlorophenoxy) acetic acid; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase; ROS, reactive oxygen species.

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[15], and copper [16]. ROS were reported to enhance gene expression of antioxidant enzymes including glutathione *S*-transferases, peroxidases, superoxide dismutases, and catalases, as well as stress-responsive proteins such as heat shock proteins and pathogenesis-related proteins [17–20]. ROS also induced certain CYPs including CYP71B6, CYP76C2, CYP81D8, CYP81D11, CYP83B1, CYP710A1, and CYP706A1 [21]. These CYP genes have ACGT-motif and/or TGA-boxes which are the core sequences of the *activation sequence-1* (*as-1*) in their promoter regions. The *as-1* is known as an ROS-responsive element as well as a salicylic acid-responsive element [22,23]. However, it remains unknown whether *as-1* is involved in xenobiotic-inducible expression of *CYP* genes in higher plants.

Tobacco CYP71AH11 metabolized the phenylurea herbicide chlorotoluron to give the *N*-demethylated and ring-methyl hydroxylated forms [24]. The transcription of *CYP71AH11* in tobacco cells was induced by 2,4-D, 1,8-naphthalic anhydride, methyl jasmonate, salicylic acid, and arachidonic acid [5]. In this study, it was attempted to clone a 5′-flanking region of *CYP71AH11* and to characterize *cis*-elements and nuclear proteins regulating the herbicide-inducible expression of *CYP71AH11*. The results obtained seem to be important for understanding molecular mechanisms of regulation of the *CYP* gene related to herbicide tolerance and selectivity in higher plants.

2. Materials and methods

2.1. Chemicals and biochemicals

3,5-Dibromo-4-hydroxybenzonitrile (bromoxynil) was purchased from Kanto Chemical (Tokyo, Japan). 5-(2-Chloro-α,α,α-trifluoro-*p*-tolyloxy)-2-nitrobenzoic acid (acifluorfen) and (9-[2-carboxy-3,4,5,6-tetrachlorophenyl]-2,4,5,7-tetraiodo-3,6-dihydroxyxanthylium dipotassium salt (rose bengal) were purchased from Hayashi Pure Chemical (Tokyo, Japan) and Chroma-Gesellschaft Schmid (Muenster, Germany), respectively. *N*-(3-Chloro-4-methylphenyl)-*N*,*N*-dimethylurea (chlorotoluron) was purchased from Riedel-de Haën AG (Seelze, Germany). 6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine (simazine), 6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine (atrazine), 2,4-D, and 1,1′-dimethyl-4,4′-bipyridinium dichloride (paraquat) were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals including β-carotene, ascorbate, ethylenediaminetetraacetic acid (EDTA), and dithiothreitol (DTT) were purchased from Sigma–Aldrich (St. Louis, MO), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemicals.

2.2. Cloning of a 5′-flanking region of tobacco CYP71AH11

A genomic DNA library of *Nicotiana tabacum* cv. Xanthi constructed in the λ-EMBL3 vector was purchased from Clontech (Palo Alto, CA). Approximately 5×10^5 phage plaques were screened by plaque hybridization using *CYP71AH11* cDNA (GenBank Accession No. GU590869) as a probe. Probe preparation, hybridization, and detection were performed with a PCR DIG Probe Synthesis Kit and a DIG Luminescent Detection Kit (Behringer, Mannheim, Germany). λDNA was isolated from positive clones with Qiagen Lamda Protocols (Qiagen, Hilden, Germany), and characterized by restriction enzyme mapping and southern blotting. A DNA fragment containing a 5′-flanking region of *CYP71AH11* was subcloned into pUC18 (Takara, Shiga, Japan) and its DNA sequence was determined by the use of a 5500L DNA sequencer (Hitachi, Yokohama, Japan). Putative *cis*-elements were searched in PLACE database [25].

2.3. Plasmid construction

The restriction enzyme *Nco*I site was added to 3′-end of a 5′-flanking region of *CYP71AH11* by PCR, and then amplified fragments were digested by *Nco*I, blunted, and ligated with a *Sma*I linker (Takara). The resulting plasmid was digested by *Eco*NI, *Nde*I, *Eco*RV, *Xba*I, or *Ava*II, blunted, and then ligated with a *Hin*dIII linker (Takara). A cauliflower mosaic virus (CaMV) 35S promoter in the vector pBI121 (Clontech) was replaced with each of *Hin*dIII–*Sma*I fragments, resulting in pIGAP, pIGBP, pIGCP, pIGDP, pIGEP, and pIGFP. pBI121 was digested by *Hin*dIII and *Sma*I, blunted, and then self-ligated, resulting in pIGGP.

The 1574-bp DNA fragment from −1281 to −740 bp of the 5′-flanking region of *CYP71AH11* was amplified with the primers −1281/−740-s and −1281/−740-as (Supplementary Table 1). Three kinds of mutated fragments were amplified with the forward primer Mutation-s and each of the reverse primers Mutation-as1, Mutation-as2, and Mutation-as3. PCR products of the 5′-flanking region from −1281 to −740 bp of *CYP71AH11* and each mutated fragment were annealed, and blunted with a Klenow fragment. Subsequently, blunted DNA fragments were amplified by PCR with the primers −1281/−740-s1 and −1281/−740-as1. PCR products were subcloned into pT7Blue T-vector (Takara), and mutated 5′-flanking regions of *CYP71AH11* were screened by sequencing. The 1574-bp 5′-flanking region of *CYP71AH11* in pIGAP was replaced with each of mutated 5′-flanking regions of *CYP71AH11*, resulting in pmCD9P, pmCD10P, and pmCD18P.

The region CD (−1281 to −770 bp of the 5′-flanking region of *CYP71AH11*) was amplified with the primers; −1281/−770-s and −1281/−770-as, and then inserted to pBImin35SP which contained CaMV35S minimal promoter (−46 to +12) and the reporter *GUS*, resulting in pCDmin35SP.

To construct 3xCD9, a mixture of three kinds of oligonucleotides 3xCD9-1, 3xCD9-2, and 3xCD9-3 was annealed and then the resulting double strand DNA fragments were ligated with pBImin35SP. Each three kinds of oligonucleotides below were used for 3xCD10, 3xCD17, 3xCD18, and 3xCD22, respectively; 3xCD10-1, 3xCD10-2, and 3xCD10-3; 3xCD17-1, 3xCD17-2, and 3xCD17-3; 3xCD18-1, 3xCD18-2, and 3xCD18-3; 3xCD22-1, 3xCD22-2, and 3xCD22-3. A plasmid in which three copies of CD9, CD10, CD17, CD18, or CD22 were inserted was selected by sequencing.

2.4. Plant growth and transformation

N. tabacum cv. Samsun NN, kindly provided by Dr. Y. Ohashi (National Institute of Agrobiological Sciences, Tsukuba, Japan), was sterilely grown in Murashige and Skoog (MS) medium containing 3% (w/v) sucrose and 0.7% (w/v) agar at 25 °C under 16 h-light and 8 h-dark photoperiods. Tobacco transformation was performed by the leaf disc method [26] with *Rhizobium radiobactor* LBA4404 carrying each plasmid. Twenty-three individual transgenic plants for the plasmid pIGAP, twenty-five for pIGBP, seventeen for pIGCP, eighteen for pIGDP, sixteen for pIGEP, fifteen for pIGFP, and seventeen for pIGGP were generated and selected by kanamycin resistance. Twenty-five individual transgenic plants for the plasmid pmCD9P, sixteen for pmCD10P, twenty-one for pmCD18P, and six for pCDmin35SP were obtained. The transgenic tobacco plants C#27 and C#38, in which the region C of the 5′-flanking region of *CYP71AH11*and the reporter *GUS* gene were carried, were used as the representative.

2.5. Chemical treatments

Axillary buds of the transgenic tobacco plants were sterilely grown in MS medium for 2 weeks, and then shoots were transplanted to fresh MS medium followed by another 2 week

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