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Biochemical and Molecular Mechanisms of Resistance Against Isoproturon in *Phalaris minor* : Variations in Protein and RAPD Profiles of Isoproturon Resistant and Sensitive *Phalaris minor* Biotypes

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ABSTRACT

Phalaris minor (Retz.) has developed resistance against isoproturon in northern parts of India. The biochemical and molecular mechanism(s) of resistance are not known so far. The present investigation was thus undertaken to predict some probable mechanism(s) by comparing the protein profiles of PS-II and microsomal fractions for both biotypes when treated with isoproturon as well as RAPD profiles of the genomic DNA in isoproturon resistant and sensitive biotypes of *P. minor*. Protein profiles on gradient-PAGE were same for both biotypes as well as after treatment with different levels of isoproturon suggesting induction of no new protein(s) in PS-II enriched and cytochrome P450 fractions. While RAPD analysis of genomic DNA showed difference in their banding patterns in two biotypes. This suggested that isoproturon resistance might be due to variation in many genes, which perhaps have regulatory roles.

INTRODUCTION

Phalaris minor is the major weed of wheat in north-west India. Among several herbicides used to control this weed, isoproturon was found most effective against *P. minor*. Over the years due to continuous use of the herbicide by the farmers, *P. minor* has developed resistance against isoproturon (Malik *et al.*, 1992). Resistance against substituted ureas including isoproturon in herbicide resistant biotypes of *Alopecurus myocuroides* was due to metabolic detoxification primarily through elevated expression or activity of cytochrome P_{450} monoxigenase rather than herbicide insensitivity of chloroplast target site (Prado *et al.*, 1991). The faster recovery of photosynthesis following pulse treatment with isoproturon in *P. paradoxa* plants is also indicative of better detoxification mechanisms in that species. Singh *et al.* (1998) by using piperonyl butoxide (PBO), an inhibitor of monooxygenase, demonstrated that degradation of ^{14}C isoproturon over 48 h was more rapid in the R biotype of *P. minor* and wheat, and major metabolites were hydroxyl isoproturon, monodesmethyl isoproturon, a carboxylic acid metabolite, didesmethyl isoproturon, conjugates and two unidentified metabolites. The hydroxy metabolites are present in larger concentration in R than S biotype. The rapid degradation of ^{14}C isoproturon in the R biotypes of *P. minor* in the absence of PBO indicated that resistance was governed by an increased activity of cytochrome P_{450} monooxygenase enzymes. In the present investigation, we have studied profiles of PSII enriched and microsomal fractions. RAPD profiles of genomic DNA were also carried out to understand any variations in the two *P. minor* biotypes.

MATERIALS AND METHODS

Seeds of two biotypes of *Phalaris minor* (resistant and sensitive to isoproturon) were sown in the pots. A pot culture experiment was conducted for both biotypes of *P. minor*. At two-leaf stage, the seedlings were sprayed with isoproturon, at 0.5, 1.0 and 1.5 kg/ha to compare control, low, normal

and high doses of the herbicides. Leaf samples were drawn at 7th day of treatment.

Photosystem -II Enriched Membrane Preparations

Reagents : Solution A–Homogenization buffer : 50 mM KH_2PO_4, 0.35 M KCl, 0.5 mM EDTA (pH 7.5); Solution –Resuspension buffer : 6 mM $MgCl_2$; Solution C–Tricine 50 mM, sucrose, 0.2 M, NaCl, 0.1M, $MgCl_2$ 3 mM (pH 8.0); Solution D–Solubilization buffer : (pH 6.3) MES 20 mM, $MgCl_2$, 5 mM and NaCl 15 mM.

Procedure : *Phalaris minor* leaves were chopped into small pieces and homogenized in ice-cold homogenization buffer (Solution A), filtered through 10 layer muslin cloth+1 layer cotton wool and centrifuged at 5000 g for 10 min at 4°C. Pellets are resuspended in solution B to rupture any intact plastids by osmotic shock and release chloroplast envelop membranes. Solution C was added to it and centrifuged at 5000 g for 10 min. Pellets were solubilized in solution D containing 10% triton X-100 and incubated in ice in the dark for 30 min centrifuged at 40,000 g for 30 min. Pellets were suspended in solubilization buffer (having 10% glycerol), lyophilized and stored at -80°C. All reactions were performed in ice at 4°C.

Isolation of Cytochrome P_{450} from Microsomal Enriched Fraction

Homogenization buffer : MOPS-NaOH, 0.1 M Sorbitol 0.3 M, Cysteine 0.05%, BSA 0.1% (w/v), EDTA 5 mM (pH 7.0); Resuspension buffer : 0.1 M MOPS-NaOH 1 ml, Glycerol 50% V/V; Dilution buffer Triton X-100 2% w/v, MOPS NaOH 0.1 M, $MgCl_2$ 50 mM (pH 7.0).

Procedure : All procedure was carried out on ice. Leaf tissues cut into small pieces were homogenized in mortar with pestle containing homogenization buffer (3 ml/g of leaf). The homogenate was centrifuged 20,000 g for 30 min. The supernatant was recovered, and centrifuged at 100000 g for 1 h. The pellets, defined as the microsomal fraction, were suspended in suspension buffer (It could be stored for several months under liquid nitrogen). Suspended microsomes were solubilized with an equal volume of dilution buffer, stirred for 15 min and then 1 M $MgCl_2$ was added. Centrifuged for 1 h at 100000 g; the supernatant was defined as cytochrome P_{450} (O' Keefe and Leto, 1988).

Polyacrylamide Gel Electrophoresis

PSII enriched fractions and cutochrome P_{450} preparations were analyzed for their protein profiles on SDS-PAGE and gradient SDS-PAGE (Laemmli, 1970).

DNA Isolation and RAPD Analysis

Reagents : Extraction buffer : 100 mM Tris HCl (pH 8.0), 50 mM EDTA (pH 8.0), 50 mM NaCl, CTAB (cetramide), 20%; Potassium acetate 5 M; Isopropanol; TE buffer (pH 8.0), 1 M Tris buffer, 0.5 M EDTA 2.8% NaCl; Ethanol 70%; 3M Sodium acetate (pH 5.2).

Procedure : Genomic DNA from leaves of S and R biotypes of *P. minor* was isolated employing CTAB method (Murray and Thompson, 1980). Protein and RNA were removed by treating the DNA preparations with phenol : chloroform : soamyl alcohol (25 : 24 : 1) and RNase, respectively. Ten random wheat primers as detailed below were got custom synthesized from Genei, India.

Operon code	Sequence (5'–3')	GC content (%)
UBC 18	GGGCCGTTTA	60
UBC 535	CCACCAACAG	60
UBC 337	TCCCGAACCG	70
UBC 552	CTAAATGGCG	50
UBC 600	GAAGAACCGC	50
UBC 532	TTGAGACAGG	50
UBC 572	TTCGACCATC	50
UBC 534	CACCCCCTGC	80
UBC 550	TGACGCGCTC	70
UBC 386	TGTAAGCTCG	50

Amplification of genomic DNA was performed for 45 cycles by employing denaturing temperature

of 94°C, annealing temperature of 34.3°C and polymerization temperature of 72°C. Denaturation time was 3 min for first cycle and then one minute for subsequent cycles. Annealing and polymerization times were 2 min for each cycle. Submerged gel electrophoresis unit was for fractionating RAPD markers on agarose gel.

RESULTS AND DISCUSSION

Resistant plants grew normally at low isoproturon levels and the growth of susceptible plants was stunted, which became apparent after one week of isoproturon treatment. Susceptible biotype did not survive after 45 days after sowing when treated with isoproturon at 1.5 kg per hectare.

Protein Profiles

PS II enriched fraction : The 5 to 15% gradient SDS-P AGE of PS II enriched fraction resolved six major polypeptides of molecular weight 60.25, 50.1, 28.84, 22.9, 21.87, 17.3 kDa and one 57.54 kDa minor band (Fig. 1). This banding pattern was similar with S and R biotypes. The protein profiles of PSII enriched fractions reveal that isoproturon does not induce synthesis of any new protein(s). However, the fact that modification of D1 protein could be a mechanism of resistance against isoproturon may still be valid because a point mutation in the gene that leads to one or few amino acids change in protein structure or chemical modification of the protein may not be significant to be detected by PAGE.

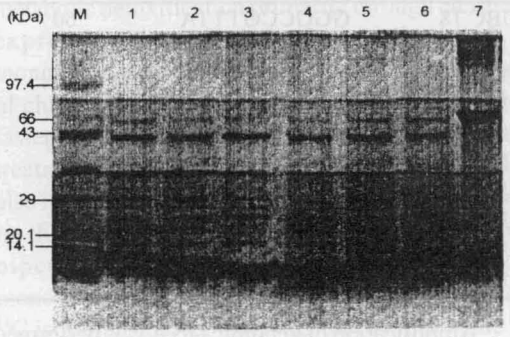

Fig. 1. Gradient PAGE for PSII enriched membrane proteins (7.2-15% gel).

Cytochrome P_{450} profiles : The SDS-PAGE of microsomal proteins on a 7% gel revealed one major sharp band with Rm value 0.941 (≈ 45 kDa) and two diffused broad bands of Rm values 0.691 (≈ 66 kDa) and 0.22 (≈ 97 kDa). Since Cyt P_{450} also has a molecular weight of around 45 kDa (O' Keefe and Leto, 1988), the sharp band at Rm value 0.941 could be of cytochrome P_{450}. This band had the same intensity in both S and R biotypes. Isoproturon treatment did not causc any change in the intensity of this band as well as of other bands. The gradient SDS-PAGE of cytochrome P_{450} revealed a single minor band of Rm value of 0.361 on 7.2% gel and two bands in 9% gel with Rm values 0.489 and 0.680. In 12 and 15% region clear separation of proteins could not be achieved (Fig. 2). The bands observed in 7.2 and 9.0% regions of gradient SDS-PAGE corresponded to, respectively, 97, 66 and 45 kDa out of which 45 kDa band was broad and prominent. Isoproturon treatment or nature of biotype did not cause any variation. The cytochrome P_{450} profile revealed that isoproturon resistant and sensitive *P. minor* biotypes had no difference. Isoproturon treatment did not cause any change.

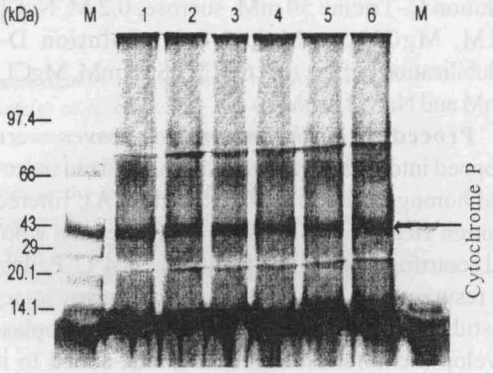

Fig. 2. Gradient PAGE of microsomal proteins (7.2-15% gel).

RAPD Profiles of Genomic DNA

The banding pattern of two biotypes of *P. minor* showed large differencc in their RAPD profile.

Out of 10 primers, only four primers showed total 15 RAPD bands. These were UBC 18, UBC 535, UBC 532 and UBC 572. Primers UBC 18 and UBC 535 scored six RAPD bands, while UBC 532 and UBC 572 scored 2 and 1 RAPD loci, respectively (Fig. 3 A and B). The differences in banding patterns were observed in both biotypes of *P. minor*. These differences in banding patterns with different primers indicate that resistance in. *P. minor* is not due to single gene, it may be due to more than one

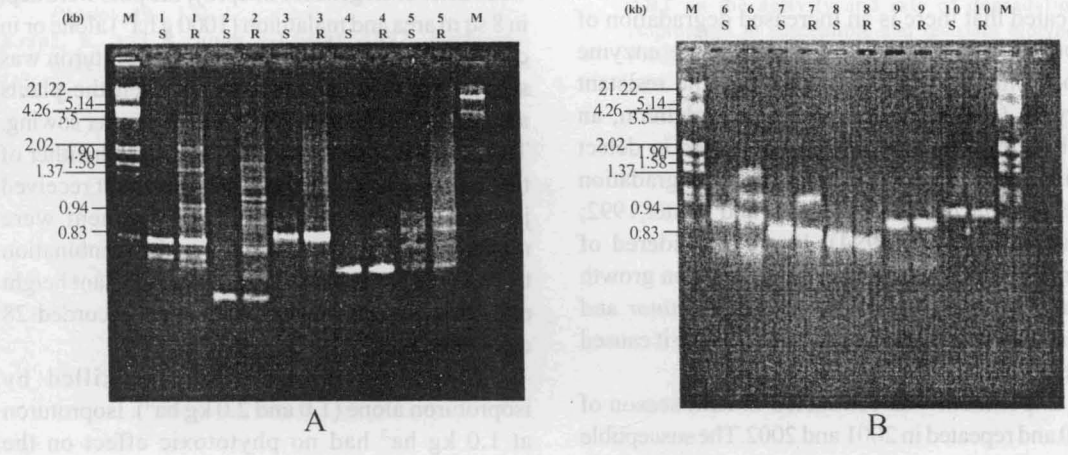

Fig. 3. RAPD analysis with wheat primers gave variations in banding patterns suggesting differences in the genomic DNAs of two biotypes. 1. UBC 18, 2. UBC 535, 3. UBC 337, 4. UBC 552, 5. UBC 660, 6. UBC 532, 7. UBC 572, 8. UBC 534, 9. UBC 550, 10. UBC 386.

gene(s). Change in the regulatory sequence(s) of genes cannot be ruled out, which enhance or suppress the expression of genes. Further, the total DNA of *P. minor* leaves was isolated and subjected to RAPD analysis. The data are not able to predict whether the variations in RAPD profiles of S and R biotypes are due to changes in nuclear or oganeller DNA. Separate RAPD profiles of nuclear and organeller DNAs will provide information about a possible mechanism because the psbA gene that encodes D1 protein is from chloroplast genome, while cytochrome P_{450} enzymes are encoded by the nuclear genome.

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