

## Molecular analysis for target site resistance in isoproturon resistant littleseed canarygrass (*Phalaris minor* Retz.)

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### Abstract

Herbicide-resistant weeds are a constraint to weed management in many cropping regions around the world. Littleseed canarygrass (*Phalaris minor* Retz.), the most predominant weed of wheat crop has developed resistance against isoproturon in North-Western Indo-Gangetic plains of India. The herbicide-binding region of the chloroplast *psbA* gene from a total of eight biotypes of *P. minor* resistant and susceptible to isoproturon was selectively amplified using PCR. Sequence analysis of the fragment from five herbicide-resistant biotypes and three sensitive biotypes exhibited none of the six substitutions as reported in other weed species of the D1 protein encoded by the *psbA* gene. The lack of mutations in herbicide binding region of *psbA* gene of isoproturon resistant biotypes indicated that target-site resistance mechanism does not account for resistance rather resistance to this herbicide can be attributed to non target-site resistance mechanism.

**Keywords:** Target-site resistance, *Phalaris minor*, herbicide resistance, Photosystem II, *psbA* gene

### 1. Introduction

Herbicides have dominated weed control in crop production systems in most of the crops since several decades. The strong and persistent herbicide selection pressure has resulted in evolution of herbicide-resistant populations in many weed species worldwide (I. HEAP [1]). Littleseed canarygrass (*Phalaris minor*) is the most predominant and troublesome winter season grass weed of wheat in North-Western Indo-Gangetic plains of India. This weed was not a problem before green revolution but large scale adoption of input responsive high yielding wheat varieties during green revolution led to the predominance of this weed under increased fertilization and irrigation practices (R.S. BALYAN & R.K. MALIK [2]). Isoproturon, a phenylurea herbicide, has been commercially used in India since late 1970s for the control of *P. minor* in wheat (H. S. GILL & al. [3]). Monocropping of rice-wheat and continuous use of isoproturon for 10-15 years have resulted in the evolution of resistance in *P. minor* during early 1990s (R.K. MALIK & S. SINGH [4], R.S. CHHOKAR & R.K. MALIK [5]). The occurrence of isoproturon-resistant *P. minor* in India is increasing year after year. Therefore, management strategies must be developed to prevent the selection and spread of isoproturon-resistant *P. minor*. For the effective management

of herbicide resistance in weeds, it is essential to understand the genetic and biochemical basis of resistance. Several herbicides from structurally diverse chemical groups (e.g., triazines, triazinones, ureas, uracils, biscarbamates) inhibit photosynthesis. They compete with plastoquinone (PQ) at the PQ binding site on the D1 protein within the photosystem two (PSII) complex. PSII electron transport inhibition stops NADPH and ATP production and the carbon reduction cycle, leading to carbohydrate starvation and oxidative stress (S.B. POWLES & Q. YU [6]). Previous research has shown that resistance to PSII-inhibiting herbicides is mainly due to two mechanisms: target site resistance (TSR) and non-target site resistance (NTSR), in which the TSR is the most common mechanism (S.B. POWLES & Q. YU [6], J.S. YUAN [7]). Of the numerous populations of weeds with resistance to several herbicides, it appears that most have resistance due to nucleotide substitution in the gene of the target enzyme (S.B. POWLES & Q. YU [6], H.J. BECKIE & F.J. TARDIF [8]). TSR usually results from amino acid substitutions in the D1 protein of PSII complex encoded by chloroplast *psbA* gene. There are six amino acid substitutions (Val-219-Ile, Ala-251-Val, Phe-255-Ile, Ser-264-Gly, Ser-264-Thr, Asn-266-Thr) in the D protein implicated in TSR to PSII-inhibiting herbicides (J. HIRSCHBERG & L. MCINTOSH [9], L.W. MENGISTU [10], J.G. MASABNI & B.H. ZANDSTRA [11], K.W. PARK & C.A. MALLORY-SMITH [12], E. MECHANT & al. [13], A. PEREZ-JONES & al. [14]). The present study was undertaken to investigate the target-site resistance mechanism in *P. minor* as reported in other weeds.

## 2. Material and Methods

### Plant material

Seeds of eight populations of *P. minor* showing differential response to isoproturon herbicide collected from farmer fields of Haryana and Punjab states of India were selected for this study. The pots were filled with soil and well-rotted farm yard manure in 6:1 ratio (v/v). Fifty seeds in each pot of *P. minor* populations were sown and plants were thinned two weeks after emergence to maintain 15 plants per pot. After thirty days of sowing, isoproturon at 0 (water only), 250, 500 and 1000 g/ha was sprayed using 350 liter of water/ha to identify the resistant and susceptible population. The spraying was done with Knapsack sprayer fitted with flatfan nozzle calibrated to deliver 350 liter of solution/ha. In order to obtain leaf samples for DNA extraction from susceptible populations, a control condition (without isoproturon) was also set aside for each population in which herbicide was not sprayed. Leaf samples of resistant population were collected from plants surviving at highest dose of isoproturon.

### DNA extraction and *psbA* gene sequencing

Genomic DNA was extracted from leaves pooled from three plants of each population using CTAB method (J.J. DOYLE & J.L. DOYLE [15]). A pair of primers (*psbAF* 5'-TGATTGTATTCCAGGCAGAGC-3' and *psbAR* 5'-ATGGAACCTTCAACAGCAGCT-3') was designed based on homologous regions of sequences available from GenBank for the chloroplast

*psbA* gene of, *Arabidopsis thaliana* (accession no. X79898), *Bromus tectorum* (accession no. AY744775) and *Poa annua* (accession no. AF131886).

A polymerase chain reaction (PCR) was performed in a 25- $\mu$ L volume containing 100 ng of genomic DNA, 2.5  $\mu$ L of 10 $\times$  *Taq* PCR buffer, 1.5 mM of MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP, 0.2  $\mu$ M of each primer and 1.0 unit of *Taq* DNA polymerase. The thermocycling program consisted of an initial denaturation at 94°C for 4 min, followed by 30 cycles of 45 sec at 94°C, 30 sec at 60°C, 60 sec at 72°C and a final cycle of 5 min at 72°C in S 1000 Thermal Cycler (Bio-Rad). The amplified product were separated by electrophoresis on 2% (w/v) agarose gel and stained with ethidium bromide and analyzed under UV light.

The amplicons were purified using HiPurATM agarose gel DNA purification spin kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India) according to manufacturer's instructions and sequenced with primers used for amplification from both the directions to minimize the errors due to sequencing artifacts. The sequencing was performed by Eurofins Genomics India Pvt. Ltd, Bangalore, India). The sequence alignments were done using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) with default parameters.

### 3. Results and discussion

The bioassay study of eight populations of *P. minor* against isoproturon revealed differential response. Three biotypes were controlled at 250 g/ha and were identified as susceptible populations. The other five populations were resistant and were having only partial or no control at isoproturon 1000 g/ha. DNA fragments of the herbicide binding region of *psbA* gene from resistant and sensitive biotypes were amplified by PCR and sequenced from both ends. The nucleotide sequences were deposited in the GenBank database (accession No. KP058502 to KP058508) of National Center of Biotechnology Information (NCBI). The derived amino acid sequence positions described in the following results refer to the full-length sequence of plastid *psbA* gene from *Arabidopsis thaliana* (GenBank accession No. X79898). Sequence analysis of the fragment from five herbicide-resistant biotypes and three sensitive biotypes exhibited none of the six substitutions as reported in other weed species of the D1 protein encoded by the *psbA* gene. The deduced amino acid sequence for *P. minor* fragment was identical to those from corresponding regions of the *psbA* gene of *P. annua* and *B. tectorum*. Amino acid sequences for *Senecio vulgaris*, *Capsella bursa-pastoris*, *Amaranthus powellii* and *Amaranthus retroflexus* differed from *P. minor* at residue 238, coding for arginine instead of lysine. The *P. minor* sequence also differed from *Amaranthus powellii* and *Amaranthus retroflexus* at residue 281, coding for valine instead of isoleucine (Fig 1). A point mutation (Ser-264-Gly substitution) in the maternally inherited chloroplastic *psbA* gene encoding the D1 protein in photosystem two (PSII) complex has been reported to be associated with PSII-inhibiting herbicides resistance in several weed species. Besides this mutation, five more mutations (Val-219-Ile, Ala-251-Val, Phe-255-Ile, Ser-264-Thr and Asn-266-Thr) have been reported in a small number of weed species [6]. Based on the model of herbicide-binding region in PS II, 17 amino acids (Phe211, Met214, His215, Leu218, Val219, Thr237, Ile248, Ala251, His252, Phe255, Gly256, Ala263, Ser264, Phe265, Asn266, Ser268 and Leu275) of the *psbA* gene are in contact with the Q<sub>B</sub> binding site (K.G. TIETJEN & al. [16]). Thus, we speculate the isoproturon resistance in *P. minor* may be due to non-target-site herbicide resistance mechanisms and herbicide resistance can be due to increased rates of herbicide metabolism. Four gene families: cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), glycosyltransferases and ABC transporters have



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