EPSPs mutation and alleles presence in glyphosate resistant D*igitaria insularis*

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ABSTRACT: There are several mechanisms of weed resistance, mostly regulated by genetic alterations in the herbicide action site. In this study, *D. insularis* cDNA from resistant and susceptible plants helped finding mutations and gene alleles. The RNA was isolated and specific primers amplified the EPSPS gene sequence cDNA. Protein structural differences of biotypes were obtained using translated amino acid sequences. Proline to threonine and tyrosine to cysteine substitution decrease EPSPS affinity for glyphosate due to aromatic ring loss. With this, some molecular mechanisms used by *D. insularis* to decrease glyphosate binding were found.

Key words: Herbicide resistance, protein structure.

INTRODUCTION

Herbicide usage is the most used for controlling weeds in agriculture fields. Glyphosate is a non-selective herbicide which inhibits *EPSPS* gene (5-enolpyruvylshikimate 3-phosphate synthase). Glyphosate became exclusive and frequently used with the implementation of GMO glyphosate tolerant crops and non-tillage sowing (WOODBURN, 2000). Weed resistance can be defined as a natural and inheritable capacity of biotypes in a population to survive and reproduce after an exposure to herbicides, which would kill plants (susceptible biotypes) in the same species (WSSA, 1998). In Brazil, glyphosate resistance was recently described in sourgrass (*D. insularis*) plants (CARVALHO, et al 2012).

Several plant mechanisms are known to provide resistance to herbicides. Resistant plants can also present more than one mechanism. In sourgrass, the *EPSPs* mutations were found in 182 and 310 positions. In this plant, non-target glyphosate resistance was also observed (CARVALHO et al., 2012). According to Gaines et al. (2013), the presence of double mutations in *EPSPS* can be related to its overexpression and proposes two loci for *EPSPS*

gene, which cause different expression. The aim of this study was to understand resistance in *D. insularis* plants by analyzing double mutations, alleles and protein structure.

MATERIALS AND METHODS

Plant material

Seeds from glyphosate-resistant *D. insularis* plants ("R") were collected from citrus culture located in the city of "Matão". Also, seeds of *D. insularis* were harvested from natural areas untreated with herbicides ("S") from lettuce areas located in the city of "Mogi das Cruzes", São Paulo, Brazil. Plants originated from these seeds were submitted to a whole-plant dose response curve.

Total RNA extraction and cDNA synthesis

Frozen tissue samples of 1.0 g were weighed and ground to fine powder in liquid nitrogen using a sterilized mortar and pestle. Total RNA extraction was performed in frozen tissue samples of 1.0 g following TRIzol® protocol (Life Technologies, USA). 1 µl of each extraction was analyzed in a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA) and only RNA samples with 260/280 ratio close to 2.0 were used for subsequent analyses. 4 µg of total RNA from each sample was treated with DNAse I (Promega, USA). 0.5 µl of each treated sample was analyzed in agarose gels, all displaying clear bands corresponding to rRNA, absence of DNA and no degradation cDNA samples were synthesized from 1.0 µg of the treated RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Life Technologies, USA) according to the manufacturer´s instructions.

Sequence analysis

Primers for EPSPS were taken from Perez-Jones et al. (2007) to perform PCR using cDNA samples. Bands with the amplicons with specified sizes were sliced, purified with ,sequenced , edited and blasted using blastx to confirm their percentage amino acid similarity to the conserved domains and were translated (http://web.expasy.org/translate/) to amino acid sequences finding the correct open reading frame (ORF), which were submitted to PFAM search (http://pfam.sanger.ac.uk/search) (Sanger Institute, England) to check the presence of each gene´s canonical protein domains and for subsequent analysis.

Digitaria insularis EPSPS protein modeling

The three-dimensional (3D) structures of the EPSPS proteins were generated by the MODELLER program (salilab.org/modweb) (PIEPER et al., 2011) to obtain the .pbd file, using translated sequences from one resistant and one susceptible cDNAs. PDB file was edited in

PyMOL program following Funke et al (2009) reported crystal structure of *Escherichia coli* (PDB identifier 3FJX) PDBsum structural analyses and Ramachandran plot statistics (http://www.ebi.ac.uk/pdbsum/) were obtain for statistical validation of the models.

RESULTS AND DISCUSSION

EPSPS gene sequencing

The resistance factor value between biotypes was 2.36. The highest similarities for cDNA were obtained for *Oryza sativa* (90%) and *Eleusine indica* (94%). The predicted proteins of all cDNA sequences were searched using the NCBI BLASTp program and showed similarity higher than 80% for all sequences.

Mutations in resistant plants and EPSPS gene alleles

The comparison of cDNA sequences in resistant plants of EPSPS gene showed two particular mutations, with nucleotide substitution of (1) cytosine to adenine in the 43 nucleotide (first position of codon), resulting the 15 amino acid in a proline to threonine change and (2) adenine to guanine in the 428 nucleotide (second position of codon), resulting the 143 amino acid in a tyrosine to cysteine change (considering position 1 as the "EVQL" amino acids) as reported by de Carvalho et al (2012) in *D. insularis*. cDNA sequencing from resistant plants of D. insularis revealed two alleles for each plant and when translated into protein exhibited at least one allele with the amino acids conferring resistance, which are threonine and cysteine. The last substitution (tyrosine to cysteine) seems to be essential to confer glyphosate resistance in *D. insularis* (Table 1). Gaines et al (2013), observed the presence of two *EPSPS* loci in susceptible *A. palmeri*, one of them amplifying in glyphosate-resistant.

Table 1. Alleles and their respective amino acids of different *D. insularis* resistant plants.

Resistant	Plant 1		Plant 2		Plant 3		Plant 4	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
NB 182	Proline	Thr	Proline	Thr	Proline	Proline	Proline	Thr
NB 310	Tyrosine	Cys	Cys	Cys	Tyrosine	Cys	Tyrosine	Cys
Susceptible	Plant 1		Plant 2		Plant 3		Plant 4	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
NB 182	Proline	Proline	Proline	Proline	Proline	Proline	Proline	Proline
NB 310	Tyrosine	Tyrosine	Tyrosine	Tyrosine	Tyrosine	Tyrosine	Tyrosine	Tyrosine

Protein structure between susceptible and resistant plants

The ORF of the partial DiEPSPS cDNA sequence encodes a polypeptide of 234 amino acid residues. By using the Pfam program (pfam.sanger.ac.uk), it was detected the conserved domain phosphoenolpyruvate: 3-phosphoshikimate 5-O-(1-carboxyvinyl)-transferase

regulating the major part of the DiEPSPS protein. A superfamily alignment (supfam.org) indicated that DiEPSPS belongs to the Enolpyruvate transferase (EPT) family, EPT/RPTC (RNA 3´-terminal phosphate cyclase)-like superfamily. The three-dimensional structures of the EPSPS protein in the susceptible and resistant plants were predicted using the reported crystal structure of *Escherichia coli* as a molecular model Procheck statistics and ramachandran graphics for both models showed favored regions for all residues and almost no disallowed regions.

The nucleotide changes and amino acid substitution described previously were identified in the structure (Pro-15 to Thr-15 and Tyr-15 to Cys-15). A previously report by Baerson et al (2002), showed that substitution of Pro to Ser, Leu, Thr resulted in increase enzyme affinity to bind to phosphoenol pyruvate in Salmonella typhimurium. The aromatic ring loss when substituting Pro and Tyr seems to have a significant potential when binding less to glyphosate (N-(phosphonomethyl) glycine), which allows the continuous synthesis of aromatic amino acids in the shikimate pathway (Figure 1). Glyphosate appears to occupy the binding site of the second substrate of EPSP synthase (phosphoenol pyruvate), mimicking an intermediate state of the ternary enzyme-substrates complex.

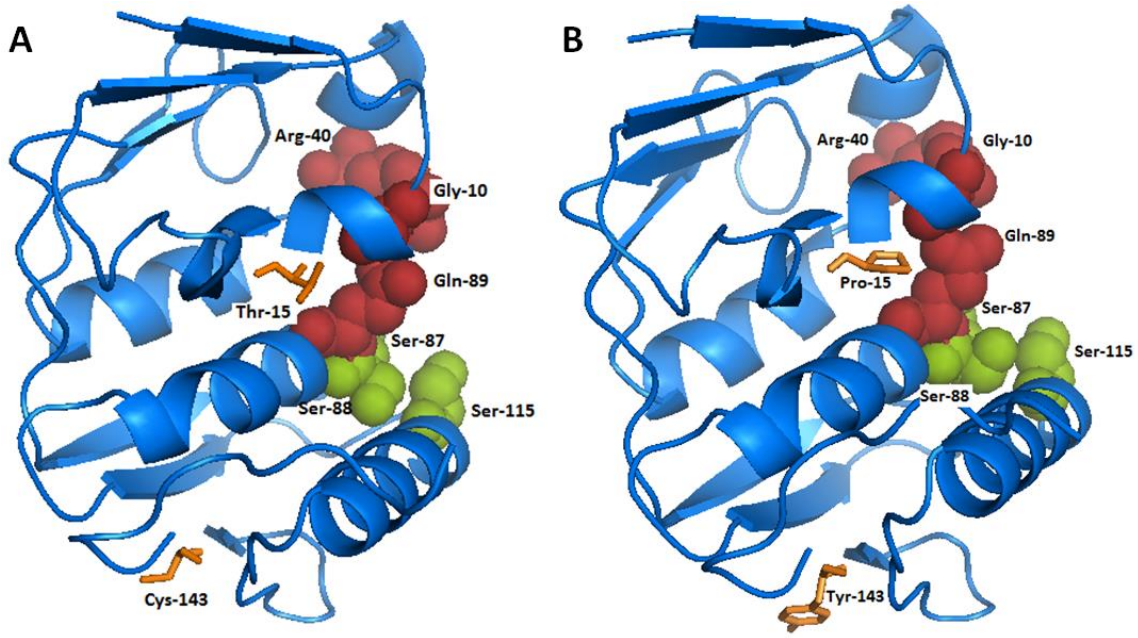

Figure 1. Structural comparison between (A) resistant (B) susceptible *EPSPS* protein predicted by MODELLER and PyMOL, using pdb file 3fjz (*E. coli*) as reference. Red and green dots represent ligation with glyphosate and shikimate-3-phosphate, respectively. Structures in orange represent amino acid substitution.

CONCLUSIONS

The results suggest that mutations in one or both alleles confer resistance to glyphosate in *D. insularis*, leading to a decrease in herbicide binding. These alterations is due a two amino acid changes consisting of a proline to threonine and tyrosine to cysteine. The protein structure losses two aromatic rings with this alteration.

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LITERATURE CITED

BAERSON, S.R. et al. Glyphosate-Resistant Goosegrass. Identification of a Mutation in the Target Enzyme 5-Enolpyruvylshikimate-3- Phosphate Synthase. **Plant Physiology**, v.129, p.1265–1275, 2002.

CARVALHO , L.B. et al. Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. **J Agr Food Chem**, v.60, p.615-622, 2012.

FUNKE, et al. Structural Basis of Glyphosate Resistance Resulting from the Double Mutation Thr973Ile and Pro1013Ser in 5-Enolpyruvylshikimate-3-phosphate Synthase from *Escherichia coli*. **Journal of Biological Chemistry**, v.284, p.9854–9860, 2009.

GAINES, T. et al. Identification of Genetic Elements Associated with *EPSPS* Gene Amplification. **PLoS One**, v.8, 2013.

PEREZ-JONES, A. et al. Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum***. Planta,** v.226, p.395-404, 2007.

PIEPER, U. et al. ModBase, a database of annotated comparative protein structure models, and associated resources. **Nucleic Acids Research**, v.39, p.465-474, 2011.

WOODBURN, A.T. Glyphosate: production, pricing and use worldwide. **Pest Manag Sci**, v.56, p.309-312, 2000.

WEED SCIENCE SOCIETY OF AMERICA. Technology Notes. **Weed Technology**, Champaign, v.2, n.4, p.789-790, 1998.