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Involvement of Epigenetic Mechanisms in Herbicide Resistance: The Case of *Conyza canadensis*

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Abstract: Glyphosate is the most important herbicide globally, and horseweed (*Conyza canadensis*) has been one of the most commonly encountered weed species that has developed resistance to it in various parts of the world, including Greece. After glyphosate application, horseweed populations show a wide range of phenotypic plasticity in response to selection pressure. In previous work, we have proposed a herbicide resistance mechanism that is not due to a point mutation at the codon 106 of EPSP synthase but most likely due to a synchronized overexpression of *EPSPS* and the *ABC* transporter genes. In the current study, it is hypothesized that the observed phenotypic alterations and differential expression of the *EPSPS* gene could be attributed to epigenetic changes. DNA methylation plays a pivotal role in many biological procedures such as gene expression, differentiation, and cellular proliferation. Sodium bisulfite sequencing was used to detect epigenetic changes that occur at the C5 position of cytosine residues within CpGdi nucleotides in two horseweed populations (resistant vs. susceptible). Results show differential methylation pattern between the two populations. This work will elucidate the naturally increased resistance of *C. Canadensis* to glyphosate and set the bases for future development of techniques that restrict weed resistance to herbicides.

Keywords: glyphosate resistance; *Conyza canadensis*; *EPSPS*; epigenetics

1. Introduction

Glyphosate is considered the world's most important herbicide. However, its prolonged use has resulted in the appearance of several biotypes of diverged weeds that have evolved resistance to the herbicide [1].

The study of resistance mechanisms in different weed species has provided valuable information regarding the evolutionary processes of weed species which were trying to combat intense selection pressure. Target-site resistance (TSR) is fulfilled by amino-acid substitutions at one of several possible positions on the herbicide target protein, causing a reduction in the efficacy of the direct action of the herbicide on its target [2–5]. Target-site mutations for glyphosate resistance are apparently rare within weed populations and not very efficient, whereas target-site mutations occurring for some other herbicides endow high levels of resistance [6]. Additionally, the recently rapid increase in the abundance and geographical extent of herbicide resistance is largely due to nontarget-site-based resistance (NTSR) which is of complex multigenic control [7,8] and cannot be explained by models used to investigate TSR. NTSR mechanisms include reduced translocation [9], vacuolar glyphosate sequestration [10], gene amplification [11], and rapid mature leaf necrosis resulting in reduced translocation [12,13].

Different glyphosate resistance mechanisms occur within populations and within individuals [14]. Several studies have already reported the synergy of both Transcriptional gene silencing (TGS) and

Non-target-site resistance (NTSR) mechanisms in order to provide higher-level of resistance [15–17]. Accumulation of multiple mechanisms is a common trend, particularly in cross-pollinated species, resulting in higher resistance levels in response to continuing glyphosate selection pressure. Consequently, efficient weed management becomes even more challenging and should utilize additional methods in order to control glyphosate-resistant weeds [18,19]. Several 'omics' approaches could help elucidating the genetic bases of complex resistances, e.g., transcriptomes are already available for several broadleaved weed species [20–22].

On the other hand, mutations are changes in a DNA sequence that can influence either TSR, causing an increase in the expression of the herbicide target protein that compensates for the herbicide inhibitory action, or NTSR by creating a variation in the expression of herbicide-metabolizing enzyme(s) or of transporter proteins that will lead to an increase in herbicide degradation or translocation, respectively [22]. Epigenetic processes (e.g., DNA methylation or histone modifications) can also be involved in the regulation of gene expression [23,24]. These modifications are not mutations in the DNA sequence, but they are transmitted to the progeny of the plant. In particular, herbicide stress is a kind of abiotic stress and triggers regulation cascades that can lead to herbicide 'stress imprint' by epigenetic modifications [24].

The epigenetic effects have the adaptive advantage of directly transmitting the 'stress imprint' of mother plants to the next generation as a pre-regulated expression pattern, without the need for mutations in regulator genes to occur, and can have a major impact on the organism capacity to respond quickly and establish a long-term stress adaptation [25,26]. Although several examples have pinpointed the potential role of cultivar-specific DNA methylation patterns as an important regulatory mechanism for sensing and responding to the stress conditions via modulation of stress-responsive genes [27,28], only a few have mentioned the implication of the aforementioned mechanisms or spontaneous mutations in the evolvement of herbicide resistance. In earlier works [29], they reported that the resistant goosegrass (*Eleusineindica* L.) biotype to dinitroaniline herbicides contains a base changes within the coding sequence of a major a-tubulin gene which is most probably due to a spontaneous deamination of methylated cytosine. Moreover, the rapid evolution of herbicide resistance in palmer amaranth (*Amaranthuspalmeri* S. Wats.) due to multiple copies of the *EPSPS* genes suggests that epigenetic responses to a changing environment based on alterations in genome architecture rather than changes in the underlying DNA base sequence could explain at least some of its capacity to adapt rapidly [30].

In our previous study, we showed that glyphosate resistance in *Conyza canadensis*, one of the most problematic weeds in terms of management in Mediterranean basin, was conferred through the synchronization of two resistance mechanisms, the overexpression of *EPSPS* gene and the simultaneous abundance of *M10, M11 ABC* transporters gene transcripts [17]. Due to the fact that *EPSPS* altered expression between resistant and susceptible biotypes was not conferred due to a point mutation, with this study, we aim to unravel the possible involvement of an epigenetic mechanism (i.e., the levels of DNA methylation of the *EPSPS1* gene in one resistant and one susceptible *C. canadensis* biotype) which could contribute to this phenomenon.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Horseweed seeds of two biotypes (progeny) previously confirmed and characterized as glyphosate resistant (R) and susceptible (S) were included in the present study [31]. The R biotype was from a citrus orchard with a long history of glyphosate use (more than 20 years) and 2–3 applications per year (Lakoniaprefecture). The S biotype's origin was from an olive orchard in Attiki prefecture that had never been treated with glyphosate and therefore was used as our reference susceptible population.

Fifty seeds from each biotype were sown in separate 12 × 13 × 5 cm pots. An herbicide-free soil from Agricultural University of Athens (AUA) mixed with a common peat substrate (1:1, *v/v*)

was used. Throughout the experiments, the pots were uniformly watered as needed and supplied with 50 mL/pot of modified Hoagland's solution (0.25%) every 10 day [32]. Seeds were placed on the substrate surface and watered. Pots were maintained in natural conditions with a photoperiod of 12–14 h and air temperatures in the range of 19–32 °C. Seedlings at the three- to five-leaf stage were transplanted into individual pots (15 cm diameter by 30 cm depth), using the same substrate.

2.2. Herbicide Treatment

Experiments were conducted on true type plants either R- (resistant) or S- (susceptible) based on previous pre-selection during other experiments [31]. When horseweed seedlings were at the rosette stage (5–10 cm diameter, 6–10 leaves), they were sprayed with 0.72 kg ae/ha of glyphosate (Roundup Gold 36SL) (maximum recommended rate) using a custom-built, compressed-air, low pressure flat-fan nozzle experimental sprayer delivering herbicide in 300 L/ha water at 250 kPa. The experiment was conducted twice. Plants were left to full developmental stage after glyphosate application (60 DAP, Days After Application) to manifest clear resistance symptoms (all plants were either resistant or susceptible). The S-plants were measured (95% control) to be effectively controlled, whereas the R-plants (22% control) were uncontrolled by the above glyphosate rate. As a standard procedure, all screening experiments routinely measured the shikimate as a biochemical screening test for glyphosate resistance [31]; the R-plants had a very low level of shikimate similar to the untreated control plants (data not shown) to further support that the used R-plants were true type glyphosate resistant ones.

2.3. DNA Isolation and Bisulfite Sequencing

Genomic DNA was isolated from 60-day-old leaves of the previously mentioned *Conyza canadensis* populations with the Nucleospin Plant II (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. DNA concentrations were determined spectrophotometrically and verified by ethidium bromide staining on agarose gels (Merck, Darmstadt, Germany). Approximately 500 ng of purified genomic DNA was used for bisulfite conversion with the EpiTect Bisulfite Kit (Qiagen, Kopenhagen, Denmark) manufacturer's instructions.

The sequence of interest was PCR (Eppendorf, Hamburg, Germany) amplified from the Bisulfite-treated DNA with a set of appropriate primers (EpspsBi-F: TATGAAATTTTGTTTTT and EpspsBi-R: CARAAACTATTAAACATTTTTTT). The amplified fragments were cloned into the pGEM-T easy vector (Promega). Around 20 individual clones were randomly selected and plasmid DNA was isolated and sequenced. The ratio of methylated to unmethylated sites was determined and the data were presented as percentages according to $100 \times C/(C + T)$.

2.4. Statistical and In Silico Analysis

For the statistical analysis of measurements the SPSS (IBM statistics v.20, IBM cooroporation, New York, NY, USA) program was used. In silico analysis of the EPSPS DNA fragment was performed using the PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), the Jaspar database (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl?rm=browse&db=core&tax_group=plants) and the PLACE database (http://ppdb.agr.gifu-u.ac.jp/ppdb/cgi-bin/all_place_motif_list.cgi?organism=At).

3. Results

3.1. Differential Epigenetic Landscape in R and S Populations of Conyza canadensis

The application of glyphosate in the R and S populations showed that the R population has developed a resistance mechanism that enables horseweed to withstand even high concentrations of glyphosate. Recent findings have shown that between the two populations there is a differential expression of the *EPSP-synthase 1* gene [17]. These differences are not relying on the mutation on

the proline 106 of the gene's nucleotide sequence. Moreover, the two populations have been shown to present modified patterns of shikimate accumulation [17].

These data prompt us to consider that there are epigenetic modifications affecting *EPSP-synthase 1* gene expression between the R and S populations. Sequenced *EPSPS1* gene from both resistant and susceptible biotypes did not reveal any change in its nucleotide sequence (data not shown). Thus the modifications could be due to the presence of methylgroups on the cytosine residues of the gene's nucleotide sequence. In order to uncover the presence of these epigenetic modifications, sodium bisulfite sequencing was performed on a specific genomic region of the *EPSP-synthase 1* gene that contains the translation start site (Figure 1). Analysis of the methylated versus un-methylated cytosine residues of the DNA sequence between the two samples showed us that in the R population the levels of the methylated cytosine residues were higher (average value of 31.1%) than the comparable ones on the S population (mean value 16.1%) (Figure 2). Even though some cytosine residues were over-methylated in the S population compared to R population (e.g., cytosine residue position 275; 71.5% in S versus 33.5% in R), the majority of the residues were over-methylated in the R population (33.5–45%) versus low methylation profiles in the S population (28%) (Figure 2).

Figure 1. Nucleotide sequence of the genomic DNA fragment of the *EPSP-synthase 1* gene of *Conyza canadensis*. Bases are numbered relative to the translation start site (red line) at position +1. Cytosine residues are marked with asterisks. Black solid lines indicate the primers used for amplification and sequencing.
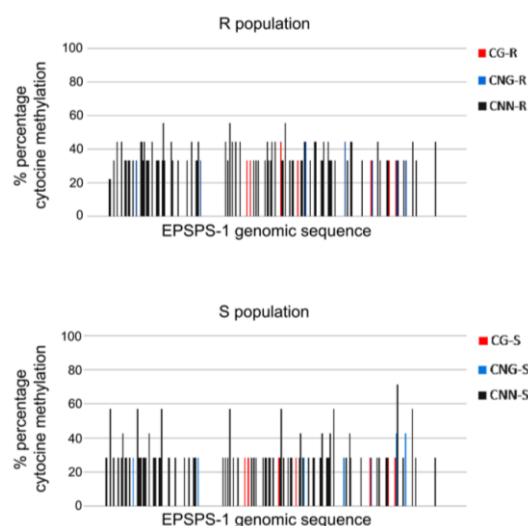

Figure 2. Bisulfite sequencing data shown as percentage methylation in CG, CNG, and CNN islands of the genomic DNA fragment of the *EPSP-synthase 1* gene of *Conyza canadensis*. Vertical lines indicate the positions of cytosines. The height of each line represents the percentage of 5′-Me-C at that position. The horizontal line of the *x*-axis on each graph represents the DNA sequence of the *EPSP-synthase 1* gene. S population: susceptible population; R population: resistant population.

The differential methylation pattern was even more obvious when the total methylation percentage was assessed for each CG, CNG, and CNN island (Figure 3). Even though the methylation pattern on the CG islands showed low variation between the two populations (28% in S versus 33.5% in R population), there was an increase in the variation of the methylation pattern in CNG islands and an even greater variation in CNN islands (28–33% in S versus 33.5–45% in R; 28% in S versus 33.5–57% in R respectively). On the contrary, only a small number of CNN islands displayed very low methylation changes in the R population in the onset of the tested DNA sequence (22%, Figures 2 and 3).
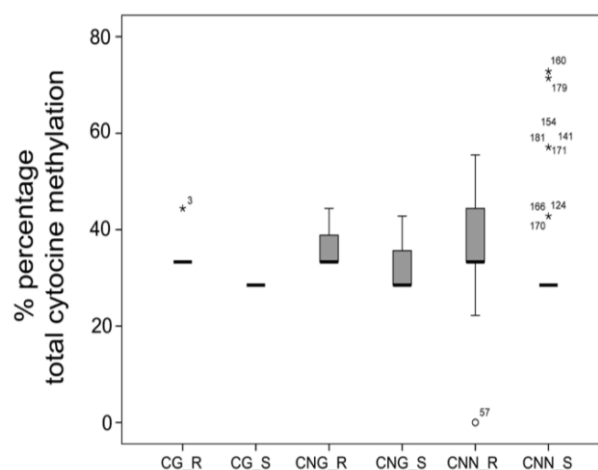

Figure 3. Total cytosine methylation changes for the CG, CNG, and CNN islands of the genomic sequence of the *EPSP-synthase 1* gene for the S and R populations. Each boxplot represents the percentage of 5′-Me-C at each island, respectively. * represents extreme values more than 3.5× of the interquartile range of the box which contains the middle 50% of the records, ° represents extreme values more than 1.5× of the interquartile range of the box.

3.2. Various Cis-Acting Elements Are Present on Conyza canadensis Nucleotide Sequence

Since the results showed that the genomic sequence of the *EPSP-synthase 1* gene had differential methylation pattern between the two populations, we attempted to identify cis-acting elements that could be affected by the presence of methyl-groups on the cytosine residues. *Conyza canadensis* is not a fully sequenced organism and although it is a weed of great economic importance, we still have limited knowledge regarding its genome sequence. Therefore, we performed in silico analysis in databases which contain information of cis-acting elements of interrelated or model organisms in order to uncover similar elements on *EPSP-synthase 1* gene's genomic sequence. As it is shown on Figure 4, the analyzed genomic sequence contains at least four different consensuses. The search revealed four CAAT-boxes that are related with promoter efficiency, two different elements that are related with light responsiveness; one element that is related with the binding of MADS-box transcription factors which regulate vegetative phase change, flowering time, branching, and leaf initiation rate; and one element that is related with abscisic acid and stress response. These bioinformatic data showed that the analyzed genomic region has significant importance for the *EPSP-synthase 1* gene's expression and subsequent pathway regulation.

Figure 4. In silico analysis of the DNA sequence of the *EPSP-synthase 1* gene of *Conyza canadensis*. Black solid lines represent the CAAT-boxes and yellow markers represent the elements found in the sequence. The red line represents the translation start site. Sp1: light responsiveness element; WRKY40: WRKY-domain transcription factor, ACE: light responsiveness element; AP1: APETALA 1 transcription factor.

4. Discussion

Weed resistance to herbicides is rapidly increasing worldwide and threatening crop security through enormous pesticide application [1]. The understanding of the genetic bases and evolutionary mechanisms of herbicide resistance is of great interest in order to highlight the complex nature of this adaptive trait and reveal potential solutions. Even though target-site-resistance can confer and, many times, explain resistance to herbicides [15,16] the recent rapid increase in the abundance and geographical extent of herbicide resistance is largely due to non-target-site resistance which is of complex multigenic control [3,6,10,19].

Epigenetic processes can be also involved in the regulation of gene expression [24]. Consequently, in the presence of either regulatory mutations or epigenetic regulations, the investigation and detection of herbicide resistance is mostly achieved by analyzing significant differences in the expression of genes between resistant and sensitive populations [22].

Conyza canadensis is one of the most challenging weeds to manage in the Mediterranean basin because of its established resistance to glyphosate [33]. In order to elucidate the resistance mechanism of horseweed to glyphosate, we examined the presence and variation of epigenetic modifications on one resistant (R) and one susceptible (S) population. The results of the bisulfite sequencing showed considerably interesting findings. The nucleotide sequence of the *EPSP-synthase 1* gene encompassing the translation start site was more methylated in the R population than the S one. Previous results [34] have shown that in R population the expression of the *EPSP-synthase 1* gene is increased. Combining the recent findings, we speculate that the increased expression of the gene is correlated with its increased methylation status.

There is evidence for a mechanistic relationship between DNA methylation of the transcribed region of a gene and the regulation of its expression in a positive correlated manner [35–37], demonstrating a novel role of gene body methylation in the eukaryotic genomes. Methylation

in the body of the gene does not block and could even stimulate transcription elongation [36]. Literature suggests that body methylation is predominately distributed in exons and enhances accurate splicing of primary transcripts [37–40]. Findings indicate that DNA methylation within the body of *Arabidopsis* genes is a key factor that may determine or negatively influence the capacity of genes to respond to internal or external stimuli (regulation of gene expression) [35]. For example, *ASR1* gene of tomato is upregulated during drought stress due to higher GC methylation levels in the first exon of the gene [41]. Linking the acquired information with our results in the differential methylation pattern between the two horseweed populations, there appears to be a good probability that the increased body methylation of *EPSP-synthase 1* gene in R population, could affect positively its expression and therefore *Conyza canadensis* resistance to glyphosate. Even though our results are preliminary, they set a sufficient base for further research to understand and clarify glyphosate resistance in *Conyza canadensis*.

Epigenetic processes are involved in the regulation of stress responses [3,23,42] and the application of herbicides is one of them. Transmission of pre-regulated expression patterns to the progeny of plants having survived herbicide stress without the need for mutations could accelerate the evolution of resistance. This topic opens entirely new research avenues for studies of herbicide resistance.

5. Conclusions

The plethora of herbicide resistance mechanisms as well as the rapid increase of weeds resistant to herbicides have multiplied the difficulties that scientists face in order to provide a well- organized weed control. On the other hand epigenetic mechanisms can alter the response of plants to adverse environmental factors and can contribute to their stress adaptation. Additionally, there are several findings supporting the idea that herbicides can induce epigenetic responses. If epigenetic effects are triggered by herbicide treatment, this will result in fast 'memory' to herbicide treatments and it will consequently fasten the evolution of resistance, particularly NTSR. Our results provide evidence that such epigenetic effects after herbicide treatment do exist and should be studied more thoroughly in the future for more efficient weed management.

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