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Detection of Target-Site Herbicide Resistance in the Common Ragweed: Nucleotide Polymorphism Genotyping by Targeted Amplicon Sequencing

Barbara Kutasy [1], Zoltán Farkas [2], Balázs Kolics [1], Kincső Decsi [1], Géza Hegedűs [3], Judit Kovács [4], János Taller [1], Zoltán Tóth [2], Nikoletta Kálmán [5], Gabriella Kazinczi [6] and Eszter Virág [1,*]

1 Department of Plant Science and Biotechnology, Georgikon Campus, Szent István University, 7 Festetics Str., 8360 Keszthely, Hungary; kutasybarbara@gmail.com (B.K.); bkolics@gmail.com (B.K.); decsikincso@gmail.com (K.D.); taller.janos@szie.hu (J.T.)
2 Department of Crop Production and Land Use, Georgikon Campus, Szent István University, 7 Festetics Str., 8360 Keszthely, Hungary; farkas.zoltan@szie.hu (Z.F.); toth.zoltan@szie.hu (Z.T.)
3 Department of Economic Methodology, Georgikon Campus, Szent István University, 16 Deák Ferenc Str., 8360 Keszthely, Hungary; hegedus@educomat.hu
4 Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Vienna Biocenter, Dr. Bohr-Gasse 3, 1030 Vienna, Austria; judit.kovacs@gmi.oeaw.ac.at
5 Department of Biochemistry and Medical Chemistry, University of Pécs Medical School, 12 Szigeti Str., 7624 Pécs, Hungary; knikoletta@gmail.com
6 Institute of Plant Science, Kaposvár Campus, Szent István University, 40 Guba S. Str., 7400 Kaposvár, Hungary; pacseszakne.kazinczi.gabriella@szie.hu
* Correspondence: virag.eszter.andrea@szie.hu

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Abstract: Background: The spread of herbicide-resistance *Ambrosia artemisiifolia* threatens not only the production of agricultural crops, but also the composition of weed communities. The reduction of their spread would positively affect the biodiversity and beneficial weed communities in the arable habitats. Detection of resistant populations would help to reduce herbicide exposure which may contribute to the development of sustainable agroecosystems. Methods: This study focuses on the application of target-site resistance (TSR) diagnostic of *A. artemisiifolia* caused by different herbicides. We used targeted amplicon sequencing (TAS) on Illumina Miseq platform to detect amino acid changes in herbicide target enzymes of resistant and wild-type plants. Results: 16 mutation points of four enzymes targeted by four herbicide groups, such as Photosystem II (PSII), Acetohydroxyacid synthase (AHAS), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) and protoporphyrinogen IX oxidase (PPO) inhibitors have been identified in common ragweed populations, so far. All the 16 mutation points were analyzed and identified. Out of these, two mutations were detected in resistant biotypes. Conclusions: The applied next-generation sequencing-targeted amplicon sequencing (NGS-TAS) method on *A. artemisiifolia* resistant and wild-type populations enable TSR detection of large sample numbers in a single reaction. The NGS-TAS provides information about the evolved herbicide resistance that supports the integrated weed control through the reduction of herbicide exposure which may preserve ecological properties in agroecosystems.

Keywords: target-site herbicide resistance; *ahas*; *als*; *psbA*; *epsps*; targeted amplicon sequencing; *Ambrosia artemisiifolia*

1. Introduction

Extensive weed management in agricultural fields is a major threat to agroecosystem biodiversity as weed communities play a key role in maintaining heterogeneity. The total number of weed species in agricultural fields has increased since the 1950s. Although this has resulted in a more diverse composition of agricultural weeds, it has negatively affected landscape structure and land-use intensity. As a result, crop intensification methods, such

as herbicide treatment, have become critical to control weed communities to improve agricultural crop production [1]. It is important not to endanger beneficial weed species while using weed control. To define the correct management of invasive species, local environmental drivers have to be identified due to their severe effects on individual performance [2].

Common ragweed (*Ambrosia artemisiifolia* L.: Asteraceae, *A. artemisiifolia)* is a highly adaptive, invasive weed and has a negative impact on biodiversity [3,4]. Its population can be found not only in agricultural habitats where herbicides are normally used but also in field margins and paths, abandoned fields, and forest edges that could be affected by herbicide or intensified mowing [5,6]. Common ragweed has spread from North America to habitats in temperate climates such as Eurasia, Asia and Australia [7,8], and has become distributed in all countries of Southern and Eastern Europe [9–12]. In North America large common ragweed populations have extensively arisen along roadsides and arable fields as well, which was recently observed mainly in Eastern Canada [13].

Many weed scientists deliberate on the knowledge and practice gap of the perspectives and approaches in weed ecology and management [14–16]. Due to the complexity of the problem, it requires transdisciplinary. Transdisciplinary approaches contribute to the most promising technological improvements managing weed control and agroecosystem diversity and reduce the potential unwanted environmental impacts of weed management. The negative correlation between weed diversity and crop yield loss has been reported [17]. As a consequence of the diverse weed management and crop production systems, the more adaptable weedy plants may replace the invasive species and the given ecosystems benefit [15]. Therefore, a well-thought herbicide use strategy should be effective and reasonable. The weed thresholds are required to promote the usage of optimal herbicides to protect crop yields and arable habitats [18].

To improve weed management and avoid ecological damages, monitoring the different herbicide-resistance in a population seems to be a great solution to find the optimal chemical crop protection. In this study, we focus on the subject of the common ragweed chemical control. Our primary goal is to achieve the controlled eradication of ragweed or at least to maintain its low moderate level while minimizing the ecological damage caused by the applied herbicide.

Common ragweed has evolved different herbicide resistance modes, making it particularly problematic to control [19]. Herbicide resistance can be categorized into two categories based on mode of action: target-site resistance (TSR) and non-target-site resistance (NTSR) [20–22]. Here, we focus on TSR resistance, which can be caused by mutation or polymorphism that results in either increased abundance of the target protein or a structural change in the target protein that reduces affinity to the herbicide [23,24]. Structural changes are generally due one or several amino acid substitutions [25–29]. The accumulation of multiple amino acid substitutions in a target protein has been known as multiple target-site resistance (MTSR) which triggers an increased resistance level of individual plants [22,30,31]. MTSR has been detected in different weeds conferring resistance to Photosystem II (PSII) and Acetohydroxyacid synthase (AHAS) inhibitors [32–34]. Resistant genotypes proliferate in areas where herbicides are used for weed control. In order to effectively combat herbicide-resistant common ragweed, effective methods to measure the prevalence of TSR and MTSR within target populations are needed.

Four herbicide mode-of-action groups are commonly used to control *A. artemisiifolia*. These are: (i) triazines and ureas (Photosystem II (PSII) inhibitors), (ii) sulfonylureas, imidazolinones, sulfonylaminocarbonyl-triazolinones, pyrimidinylthiobenzoates and triazolopyrimidines (AHAS inhibitors), (iii) glyphosate (5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) inhibitors) and (iv) diphenylethers, N-phenylphthalimides, aryl triazolinones, phenylpyrazoles, pyrimidinediones (protoporphyrinogen IX oxidase (PPO) inhibitors).

PS II inhibitors bind to the D1 protein of the photosystem II (EC: 1.10.3.9) complex, blocking electron transport and stopping CO_2 fixation. The D1 protein is encoded by the chloroplast *psbA* gene. The first documented herbicide resistant population of *A. artemisiifolia*

was discovered in 1976 and was resistant to the PSII-inhibitor atrazine (a triazines) [35]. Resistance was conferred by a point mutation in the *psbA* gene resulting in a serine-to-glycine substitution, which alters D1 protein conformation, causing reduced herbicide target interaction [36,37]. To detect the causal mutation (Ser264Gly) in common ragweed, a bidirectional allele-specific PCR (Bi-PASA) method was developed [38]. Resistant biotypes and populations have also been reported against linuron (belonging to ureas) in eastern Canada [39,40]. TSR, conferred by the *psbA* mutation Val219Ile, was detected after linuron treatment using TaqMan single nucleotide polymorphism (SNP) genotyping and Sanger sequencing [41]. Moreover, three additional mutations at the site of action (Ala251Val, Phe255Ile, and Asn266Thr) have been confirmed in populations of other species, each with a unique cross resistance pattern [42].

AHAS inhibitors are among the most used common ragweed inhibitors worldwide. AHAS (EC 4.1.3.18 also known as acetolactate synthase, ALS) catalyses the first common step in the pathway for synthesis of the branched-chain amino acids leucine, isoleucine, and valine in plants [43]. AHAS inhibitors have low mammalian toxicity, are broad spectrum and highly effective in low doses. However, resistance to AHAS inhibitors tends to arise rapidly in weed populations. Multiple studies have reported that AHAS inhibitor-resistant weed species are more numerous than weed species resistant to other herbicide groups [44,45]. Many AHAS inhibitor-resistant common ragweed populations have been reported. Cross resistance in these populations is common, with resistant individuals displaying resistance to additional herbicides, including cloransulam-methyl (triazolopyrimidines), chlorimuron (sulfonylureas), and imazaquin (imidazolinones) [46]. Resistance in common ragweed against AHAS inhibitors is conferred by the substitution Trp574Leu [47]. Although eight different AHAS amino acid substitutions confer resistance to AHAS inhibitors in other species, only Trp574Leu has been reported in common ragweed [20,48].

Several polymorphisms in the *epsps* gene of many plant species confer resistance to glyphosate. Resistance is conferred by mutations at Pro106 and Thr102, and recently an example of MTSR was reported in *Amaranthus hybridus* that contained the triple-substitution Thr102Ile, Ala103Val and Pro106Ser [49–52]. Glyphosate resistance in *A. artemisiifolia* was first reported in Missouri and Arkansas in 2004 but the mechanism of the resistance has not yet been determined [53].

PPO inhibitors have been used widely to control weeds in different crop cultures since the 1960s. PPO (EC 1.3.3.4) is a key enzyme in the tetrapyrrole biosynthetic pathway that produces heme and chlorophyll in plastids and mitochondria [54]. Two different nuclear-encoded PPO isozymes are in plants: plastidal PPO1 encoded by the gene *ppx1* and mitochondrial PPO2 encoded by the *ppx2* gene [55]. The weed reported to evolve resistance to PPO inhibitors was *Amaranthus tuberculatus*, which contained a glycine deletion at position 210 (ΔG210) in PPX2 (GenBank accession: DQ386114) [56]. In *A. artemisiifolia*, an Arg98Leu substitution in the *ppx2* gene contributes to PPO resistance [57].

Multi-resistant *A. artemisiifolia* populations can be found worldwide, containing multiple resistance-conferring mutations in genes encoding PSII, AHAS, EPSPS, and PPO [46]. The investigation of specific mutations conferring herbicide resistance in populations is essential for the selection of effective herbicides for weed control. Targeted amplicon sequencing (TAS) is an effective approach that takes advantage of NGS (next generation sequencing) to detect specific mutations. To sequence large numbers of targeted gene regions, NGS is using indexed primers to label samples that are expansively used for population genetics and for genome-wide genotyping in plants with ultralow SNP densities [58].

This study presents an NGS-TAS method for TSR identification in *A. artemisiifolia*. In order to discover multiple polymorphisms in *psbA*, *ahas*, *epsps*, and *ppx2* that confer resistance, we analysed sensitive biotypes, as well as imazethapyr-resistant (imidazolinones, AHAS inhibitor) and linuron-resistant (ureas, PSII inhibitor) biotypes. This method may contribute to the sustainable maintenance of biological integrity in agroecosystems by screening herbicide resistant common ragweed populations.

2. Materials and Methods

2.1. Plant Materials and DNA Extraction

Leaves of 20 susceptible *A. artemisiifolia* plants were collected from an agricultural field in West-Transdanubian region of Hungary (46°44′54.7″ N 17°14′07.7″ E). Seeds of linuron resistant biotypes were collected in a field border of Ste-Clotilde Legault Quebec, Canada (45°07′14″ N 73°38′19″ W) and seeds of imazethapyr resistant biotypes were collected in a soybean field of Mirabel Quebec, Canada (45°39′16.94″ N 74°05′45.31″ W). Seedlots were sampled from sites where ragweed was tested and identified as imazethapyr and linuron resistant. Resistance seeds were germinated in petri dishes at 30 °C/20 °C 16 h photoperiod and were planted in pots and were growing in greenhouse. Leaves of 20-20 planted individuals were further collected. Samples were frozen in liquid nitrogen immediately after collection and stored at −80 °C until DNA and RNA analysis.

Total DNA from leaf tissues was isolated according to the protocol Doyle, J. J. and Doyle, J. L. [59]. The DNA quantity and purity were assessed on NanoDrop2000 (Thermo Scientific, Waltham, MA, USA). For the experiments, DNA of resistant and sensitive biotypes were used according to the following classification: (i) bulked sample of 20 sensitive genotypes deriving from Hungarian sampling site; (ii) bulked sample of 20 imazethapyr (imidazolinones) resistant genotypes collected from Mirabel sampling site (AMI) and (iii) bulked sample of 20 linuron (ureas) resistant genotypes collected from Ste-Clotilde Legault sampling site (AMU). All individuals, both susceptible and resistant genotypes, were analyzed by Sanger method.

2.2. Identification of A. artemisiifolia Herbicide Target Enzyme mRNAs

In order to identify in silico coding sequences (cds) of herbicide target enzymes of each herbicide group, we used the transcript dataset of *A. artemisiifolia*, deposited in the National Centre for Biotechnology Information's (NCBI) Transcriptome Shotgun Assembly (TSA) dataset under the accession GEZL01000001 [60]. Reference genes were selected based on taxonomic relationship from closely related species and were downloaded from the UniProt database: *A. artemisiifolia*, *psbA* (B5MF75); *Xanthium* sp., *ahas* (Q41716); *Cichorium intybus*, *ppx1* (Q9SPL6); *Amaranthus palmeri*, *ppx2* (A0A291B3V5); *Erigeron canadensis*, *epsps* (G4U4J5) [61]. The identity of reference genes and identified genes was 96–99% [62]. The investigated sequences were deposited in the NCBI GenBank database (Accession numbers see below).

2.3. Intron Analysis, Amplification and Cloning of A. artemisiifolia Herbicide Target Enzyme Genes

Multiple primer pairs were designed in order to cover all possible mutation points along the whole *psbA* and *ahas* gene sequences by using Primer3Plus [63] (Figures S1 and S2).

In the case of intron containing *epsps* gene, the exon-intron bounds were predicted based on complete gene sequences of closely related species by using ClustalW aligning the sequences of *Conyza canadensis* (AY545667.1) and *Amaranthus palmeri* (FJ861242.1 and FJ861243.1) [64] (Figure S3).

In order to validate polymorphisms that characterize herbicide resistance, the imazethapyr, linuron resistant, and sensitive bulked samples were amplified. The PCR amplifications was performed by using Dream Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) The PCR products were separated on 1.5% agarose gel (Promega, Madison, WI, USA) and were purified using NucleoSpin Gel and PCR Clean-up system (Macharey-Nagel GmbH & Co, Düren, Germany). The pGEM-T Easy Vector System kit (A1360 Promega, Madison, WI, USA) and JM109 Competent Cells were used to clone PCR products [65]. Sanger sequencing of the cloned fragments was performed with ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequenced fragments of herbicide target genes were aligned to predicted gene sequences certifying amino acid substitutions in the mutation points.

2.4. PCR Amplification for NGS-TAS Experiments

The KAPA HiFi HotStart PCR Kit (Kapa Biosystems, Wilmington, NC, USA) was used for amplification of target fragments. In NGS-TAS experiments of investigated regions of *psbA*, *ahas*, *epsps*, and *ppx2* genes, primers were designed by using Primer3Plus (Figure S7).

The PCR amplifications were performed and the PCR products were separated on 1.5% agarose gel (Promega, Madison, USA). PCR products were controlled with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using Agilent DNA 1000 Kit and were purified using NucleoSpin Gel and PCR Clean-up system (Macharey-Nagel GmbH &Co, Düren, Germany).

2.5. NGS-TAS Experiments

For NGS-TAS experiments, resistant samples were used and grouped with the following nomenclature.

Sample AMI was named based on the imidazolinone (imazethapyr) resistant bulked DNA of *A. artemisiifolia* and sample AMU was named based on the urea (linuron) resistant bulked DNA of *A. artemisiifolia*. Numbers 1–3 were three biological repeats of the same bulked DNA (AMI1-3, AMU1-3). The concentrations of PCR products were measured by NanoDrop2000 (Thermo Scientific, Waltham, USA) and fragments for each sample were diluted relative to the PCR product with the lowest concentration. AMI1control and AMU1control contained the not diluted samples of the first biological repeats. In the AMI and AMU groups bulked DNA of 20 individuals were used.

In order to obtain long fragments that cover all the mutation points in each gene, fragments were sequenced on MiSeq 550 platform obtaining 300 bp paired-end reads detailed as follows: locus-specific PCR products were purified using 1.0 volume KAPA PureBeads (F. Hoffmann-La Roche, Basel, Switzerland) according to the manufacturer's protocol. The concentration of eluted DNA was measured using a Qubit 3.0 Fluorometer with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA). Index PCR reactions (20 µL each) were set up by using 20 ng of purified template in 6 µL, 2 µL Nextera XT Index kit v2 Primers (N7xx & S5xx) (Illumina, Inc. San Diego, CA, USA), and 10 µL of 2xKAPA Hifi Hot Start Ready Mix (F. Hoffmann-La Roche, Switzerland). The used primer and IlluminaNextera adapter sequences are detailed in Figure S7. PCR cycling parameters for index PCRs were as follows: initial denaturation at 95 °C for 3 min; 8 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; final extension at 72 °C for 5 min. PCR products were purified using 1.0 volume KAPA PureBeads and eluted in 20 µL of 10 mM Tris-HCl pH 8. The product libraries were quantified and qualified by using High Sensitivity D1000 ScreenTape on TapeStation 2200 instrument (Agilent Technologies, Santa Clara, CA, USA) (Figure S11). Equimolar concentrations of libraries were pooled, diluted to 4 nM, and combined with other sample pools to gain the desired sequencing depth. Sequencing was carried out using Illumina MiSeq platform and 600-cycle Reagent Kit v3 (Illumina, Inc. San Diego, CA, USA). Samples were demultiplexed and adapter-trimmed by using MiSeq Control Software.

2.6. Bioinformatics Analysis

Pear software [66] was used to merge paired-end reads with minimum overlap size 10 bp, without discard uncalled bases, and with 30 parallel threads in processing (arguments –v 10 –u 1 –j 30). All the samples contained the reads amplified by the 7 primer pairs covering all the mutation points. Accordingly, the reads belonging to each fragment were counted in each sample (Figure S12). Usearch software [67] was applied to collect identical sequences for resistance polymorphism. Usearch was used in three steps: (i) Quality filter with fastq_filter command with fastq_maxee 1.0 and fastq_minlen 160 parameters; (ii) Singleton filter with fastq_uniques command; (iii) Grouping with cluster_otus parameter with minsize 10. Groups were shown the similarity threshold 99% representing the different amplicon products with nucleotide polymorphism for the investigated gene.

3. Results

In order to perform NGS-TAS analysis, it would be necessary to know cds and gene sequences of herbicide target enzymes. However, no whole genome sequence data of *A. artemisiifolia* yet exists. To design correct NGS-TAS primer pairs, it is essential to know at least the sequences at the intron-exon boundaries close to the mutation points. Accordingly, gene sequences and point mutations of the herbicide target enzymes were determined in sensitive genotypes by using in silico NGS transcriptome analysis (NGS-TA), SS Sanger sequencing, and NGS-TAS (Figure 1).

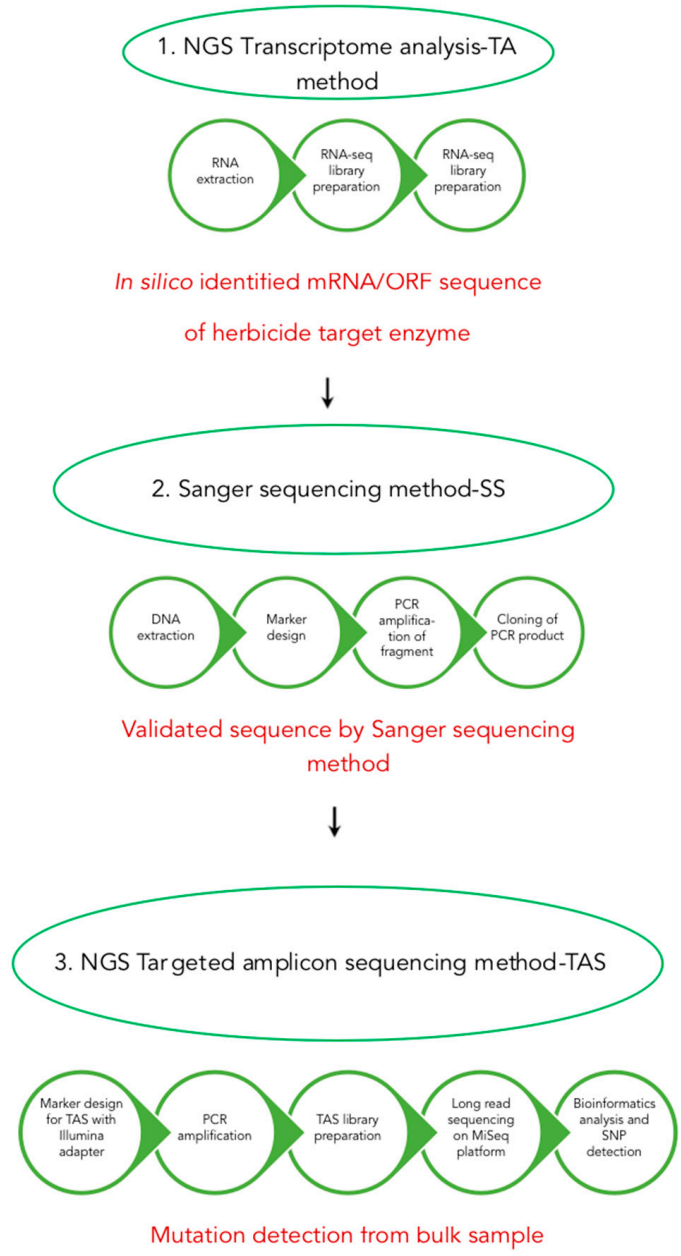

Figure 1. Flow chart of target-site resistance (TSR) diagnostic method using the following steps: (i) Next generation sequencing (NGS) Transcriptome analysis (NGS-TA). Open reading frames of target enzymes were identified based on transcriptome database; (ii) Sanger sequencing method (SS). Specific primers were designed covering the mutation points, fragments were amplified by PCR, PCR products were cloned and sequenced; (iii) Next-generation sequencing-Targeted amplicon sequencing (NGS-TAS) method. PCR products were sequenced on Illumina MiSeq550 platform resulting 300 bp paired-end reads that were sorted based on primer motifs, assembled, and grouped.

3.1. Identification of A. artemisiifolia Herbicide Target Enzyme cds and Genes

In silico analysis of investigated herbicide target enzyme cds was performed with BLAST+. The accession numbers of predicted GOIs are MT425203 (*psbA*), MK096760 (*ahas*), MK096765 (*epsps*), and MK096762 (*ppx2*). Open reading frame (ORF) sequences were predicted using NCBI ORF finder [68]. The predicted sequence of the *psbA* gene consisted of one exon with a total length of 1062bp, as previously described (Table 1) [37]. The *psbA* gene was amplified with 2 primer pairs covering the resistance mutation points as described before (Figure S1, GenBank accession MT879746).

The structure of the *ahas* gene contained one exon with a total length of 1965bp. The whole *ahas* gene was amplified with five primer pairs covering the resistance mutation points as described before (Figures S2 and S4, GenBank accession MT415954) [20,47].

The structure of the *epsps* gene contained 8 exons and 7 introns with a total length of 3539bp. A similar structure was described in *Amaranthus palmeri* (JX564536.1) [64]. The *epsps* gene was amplified with eight primer pairs covering the resistance mutation points as described before (Figures S3 and S4, GenBank accessions MT415955 (exon1-intron1), MT409110 (intron1-exon8)) [32].

The first published weed species to evolve resistance to PPO-inhibiting herbicides was *Amaranthus tuberculatus* [56]. The *ppx2* gene (GenBank accession DQ386118) from the resistant biotype of *A. tuberculatus* contained a glycine deletion at position 210 (ΔG210) that was shown to confer resistance. This deletion in *A. artemisiifolia* has not been published, yet, although the substitution Arg98Leu was discovered to contribute to PPO-inhibitor resistance *A. artemisiifolia* [57]. So far, no complete gene sequence exists of the whole *ppx2* gene in plants covering all of the introns and exons. Therefore, identifying the complete sequence of *ppx2* gene is not possible only using Sanger sequencing. By using the NGS-TAS, a 324 bp fragment of *ppx2* gene was amplified with one primer pair covering the resistance mutation point Arg98 [55]. Electropherogram of Sanger sequenced PCR product revealed two product lengths. The differences between the two fragments were 13 bp localised in one intron (Figures S4 and S5, GenBank accession MT879747, MT879748).

In order to obtain accurate information about the investigated enzymes, in silico predicted cds of Illumina data and the Sanger sequenced data of the appropriate PCR products were compared. The two technologies showed slight variability (1–2% differences) in amino acid sequences across *ahas* and *epsps*, however these discrepancies were not at mutation points. There was no difference in sequencing results of the *psbA* gene between the two methods (Figure S6).

3.2. NGS-TAS Experiments of AMI and AMU Biological Repeats

In NGS-TAS experiments specific regions of *psbA*, *ahas*, *epsps*, and *ppx2* genes were amplified by different primers (Figure 2, Figures S9 and S10). Sample AMI was named based on the imidazolinone (imazethapyr) resistant bulked DNA of *A. artemisiifolia* and sample AMU was named based on the urea (linuron) resistant bulked DNA of *A. artemisiifolia*. Both AMI and AMU contained three replicates.

Table 1. Detailed information of gene structures and coding sequences (cds) of *psbA*, *ahas*, *epsps*, and *ppx2* genes.

Gene Name	Length (bp)	Lenght (aa)	Similarity (%)	cds				Complete Gene				
				Reference ID NCBI GeneBank	Contig ID NCBI GeneBank GEZL01000001	NCBI Accession	GC Content (%)	Length (bp)	Intron Number	Intron Position from … to	NCBI Accession	GC Content (%)
psbA	1062	353	99%	*A. artemisiifolia* AB427162.1	TR92155|c0_g1_i1	MT425203	41.7	1062	-	-	MT879746	41
ahas	1965	654	95%	*Xanthium* sp. AAA74913.1	TR49503|c0_g3_i1 TR49503|c0_g8_i1	MK096760	48.1	1965	-	-	MT415954	48.3
epsps	1539	512	96%	*Helianthus annuus* XP_022017499.1	TR44247|c0_g1_i1	MK096765	45	3539	7	316 … 1311 1557 … 1832 1987 … 2114 2330 … 2410 2529 … 2656 2868 … 3006 3069 … 3400	MT415955 MT409110	34 37.5
ppx2	1476	491	95%	*Helianthus annuus* XP_021982414.1	TR33881|c0_g1_i1	MK096762	42.8	Partial 325	1	56–276	MT879747 MT879748	34.3 33.1

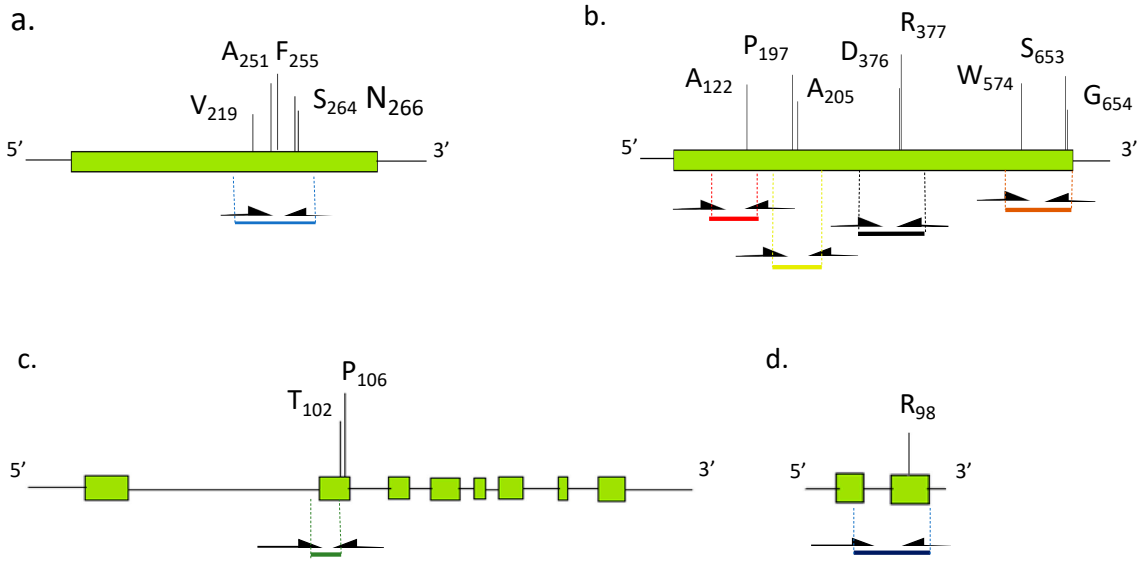

Figure 2. The schematic representation of (**a**), *psbA*; (**b**), *ahas*; (**c**), *epsps*, and (**d**), *ppx2* genes in *A. artemisiifolia* indicating regions amplified by different primers. Vertical grey lines indicate amino acid positions that contain substitutions in herbicide resistant plants. The number after the amino acid refers to the amino acid position. Green boxes indicate exons and grey lines between boxes indicate introns. Colour lines show positions of primer pairs.

Each sample contained the PCR products of *psbA*, *ahas*, *epsps*, and *ppx2* genes. The control of AMI1-3 group was labeled as AMI1control and contained PCR products of AMI1. Similarly, AMU1control served as a control for AMU1-3 group and contained PCR products of AMU1. Both in AMI and AMU samples, the concentrations of the PCR products of the four genes were measured in each replicate, respectively. Afterwards, the lowest concentration was used as a base for further dilution of the other PCR products to avoid error in sequencing analysis (Table 2, Figure S8). In NGS-TAS analyses the samples were sequenced in four lanes. Based on the ratio of concentration/contig number of the samples, we found that all concentrations of AMI and AMU samples were optimal for the diagnostic procedure.

3.3. Bioinformatics Analysis of AMI and AMU Groups

Samples with different concentrations showed different distributions in contig number (Table 2). As a result of amplicon sequencing the number of Illumina MiSeq 300 bp reads were 276,462 (forward), 296,722 (reverse) in case of AMI and 316,465 (forward), 369,748 (reverse) in case of AMU samples. After quality filtering dropped reads were 0.7% on average of AMI samples and 4.7% on average of AMU samples. PEAR software was used to assemble cleaned reads of which unpaired ones were filtered out with the average ratio 5.45 and 9.05% of AMI and AMU samples, respectively. Numbers of filtered and assembled reads were 255,357–287,197. In order to separate the investigated gene fragments we clustered the contigs (assembled reads) into groups based on appropriate primer motifs by using Usearch software. During clustering singletons were dropped (unique groups with one sequence). Average ratios of the survived contigs were 69.8% and 69.7%. The group number was 18 and 12 in AMI and AMU samples (Figure S12).

Table 2. Concentrations of PCR product (cc: ng/μL) and numbers of contig in each group in imidazolinone (imazethapyr) resistant bulked samples (AMI) and urea (linuron) resistant bulked samples (AMU).

Fragments	Length of Fragment with Adapter (bp)	AMI								AMU							
		1 Sample		2 Sample		3 Sample		1 Control Sample		1 Sample		2 Sample		3 Sample		1 Control Sample	
		cc	Number of Contig	cc	Number of Contig	cc	Number of Contig	cc	Number of Contig	cc	Number of Contig	cc	Number of Contig	cc	Number of Contig	cc	Number of Reads
psbA	377		26,989		34,594		30,560	72.1	32,592		28,106		28,523		30,492	48.3	40,178
ahas 1	288		10,009		25,817		11,802	60.3	9129		8066		21,401		12,709	24.3	11,426
ahas 2	273		45,613		32,641		45,887	50.3	34,158		43,411		47,525		50,635	43.4	52,289
ahas 3	319	50.3	18,488	32.8	15,106	40.8	23,639	74.3	20,210	24.3	25,580	19.1	21,738	19.9	19,612	37	23,140
ahas 4	453		12,563		13,668		11,955	88.6	17,567		13,133		15,549		15,854	37.1	12,032
epsps	310		21,543		34,053		29,779	51.9	16,599		18,103		10,839		17,476	31.7	15,737
ppx2	390		11,580		9280		26,659	58.8	10,546		15,205		10,108		9346	37.5	15,583

Fragments in each sample contain different numbers of contigs. Primer sorted groups contain min 8066 max 52,289 contigs (Table 2). Undiluted AMI1control and AMU1 control samples showed an equable read distribution in each fragment, therefore dilution of samples in the same concentration is recommended. Confirmation of a fragment with such a large number of reads provides an opportunity for large-sample analysis.

3.4. Detection of Mutation Points in Resistant Biotypes

To be able to identify the mutations causing resistance in *A. artemisiifolia*, bulked and amplified DNA sequences of imazethapyr and linuron resistant samples were further analysed by NGS-TAS that covered 16 known resistance mutations sites in *A. artemisiifolia*. In the AMI group, sequence alignment analysis showed that a single nucleotide in the *ahas* gene was changed from TGG to TTG and resulted in a Trp574Leu substitution conferring herbicide resistance [47] (Figure 3). Other amino acid substitutions in the D1 protein, EPSPS and PPX2 enzymes at known resistance-conferring mutations sites were not found. Based on these data, the investigated population was susceptible to PSII, EPSP synthase and PPO inhibitors (Figure 3). In the AMU group, sequence alignment analysis showed that a single triplet was changed (GTA to ATA) in the *psbA* gene and resulted in a resistance-conferring Val219Ile substitution [41] (Figure 3). Other amino acid substitutions in AHAS, EPSPS, and PPX2 enzymes at known resistance-conferring mutations sites were not found. Based on this data the investigated biotype was susceptible to AHAS, EPSP synthase, and PPO inhibitors in terms of TSR (Figure 3).

Figure 3. Amino acid mutations in investigated gene products. Imidazolinone and urea resistant bulked samples were analyzed by NGS-TAS that covered 16 known resistance-conferring mutations sites in *A. artemisiifolia*. Marking: green, possible mutation sites; red, mutant amino acids. DNA sequencing revealed that an inferred leucine for tryptophan substitution at amino acid position 574 in AHAS enzyme was responsible for the imazethapyr herbicide resistance. Amino acid substitution at the position 219 (valine/isoleucine) in *psbA* gene product, D1 protein, was responsible for the linuron resistance. Amino acid substitutions in EPSPS and PPX2 enzymes at known resistance-conferring mutations sites were not found.

4. Discussion

Chemical weed control is one of the most common ways to fight against weeds in agricultural areas, giving a high selection pressure for resistant biotypes. After herbicide treatment, the rapid diagnosis of resistance mutations present in surviving individuals using would inform and improve the effectiveness of the next herbicide treatment. In order to maintain sustainability in crop fields and beneficial weed communities that are required for natural ecosystems, the monitoring of resistant common ragweed populations between treatments is important. In this study, we present a molecular biology-based approach (NGS-TAS) to detect herbicide resistant common ragweed populations in arable habitats [69,70].

It is important that only a few herbicides with new mechanism of action (MOA) have been released since the 1980s. The increasing number of herbicide resistant weeds and the lack of discovery of new MOAs make it difficult or even impossible to use existing herbicides effectively. Some studies predict that these problems will endanger the sustainability of weed control [16,71]. Therefore, it is essential to inform farmers about integrated and resistant weed management strategies that are less harmful to the environment [16].

Determining TSR would largely improve the weed management strategy of arable lands. Field sampling of populations and testing them by NGS-TAS method would guide farmers to select the proper herbicides thereby decreasing the impact of agricultural chemical inputs on the populations of crop fields. Thereby, unnecessary and ineffective chemical usage could be avoided [16].

In this study, we describe an effective diagnostic process using NGS-TAS to get information about evolved TSR in *A. artemisiifolia* populations. In NGS-TAS, TSR is determined using SNP genotyping following genome-wide genotyping. We demonstrate that NGS-TAS analysis is a method that can monitor TSR against four different enzymes targeted by different herbicides in *A. artemisiifolia* simultaneously. In order to analyse SNPs as part of the resistance mechanism of *A. artemisiifolia*, coding sequences and complete genes of D1 protein, AHAS and EPSPS were identified. However, the sequence of PPX2 only partially was identified at the region of interest as its length and genomic data is unknown. Mutations and polymorphisms in these four proteins that had previously been reported to confer resistance were specifically investigated. The developed NGS-TAS markers can identify 16 amino acid substitutions of the investigated common ragweed genes among which 4 (*psbA*: Val219, Ser264, *ahas*: Trp 574 and *ppx2*:Arg98) were proven. As a result, the investigated samples characterized by imidazolinone (imazethapyr) and urea (linuron) TSR were proved to carry mutation points at Trp574Leu and Val219Ile in the *ahas* and *psbA* genes, respectively. Although no MTSR existed in the studied resistant samples (AMI, AMU), the NGS-TAS method can be used to detect multiple herbicide resistance, which needs to be confirmed by specific MTSR samples.

NGS technology can examine a large number of samples simultaneously using fragment or sample-specific indexed primers. Therefore, amplicon primers were provided that are suitable for discriminating mutation points. In this study, we demonstrated the trial of a pooled sample evolved mutations points of herbicide target genes supporting resistance of *A. artemisiifolia* populations and biotypes.

5. Conclusions

Invasive weed species appeared to be the bane of biodiversity, ecosystem services, and food security. Integrated weed management practices provide several comprehensive solutions for weed control to reduce weed coverage in agricultural fields. Unfortunately, many weed management approaches used nowadays are highly unsustainable. Moreover, many studies report on the rapid evolution of herbicide-resistant weed populations. This complex evolution of herbicide-resistant weeds makes it difficult to use sustainable herbicide technology [20]. Therefore, applying weed management tools for invasive plants are often ineffective at producing long-term benefits [72]. In order to map evolved resistance

in populations, different herbicide resistance mechanisms have to be investigated, such as TSR.

To summarize, NGS-TAS method is a powerful approach that was used for the detection of amino acid polymorphism and mutations that induce TSR. Therefore, it can improve effective weed control taking into account biological integrity and sustainability in agroecosystems by selecting the optimal and effective herbicide usage in cropping systems.

Supplementary Materials: The following are available online at https://www.mdpi.com/1424-2818/13/3/118/s1, Figures S1–S12: Primers, gel pictures and electropherograms of Sanger sequencing. Primers, gel pictures, electropherograms and concentrations of amplicon sequencing. Bioinformatics analysis.

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