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Genetic Variability of Acetolactate Synthase (ALS) Sequence in *Centaurea cyanus* Plants Resistant and Susceptible to Tribenuron-Methyl

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Abstract: *Centaurea cyanus*, belonging to the Asteraceae family, is an arable weed species encountered mainly in fields with cereals, sugar beet, and maize. The high genetic variability of *C. cyanus* has been recently reported; however, little is known about its sequence variability in the context of its herbicide resistance. *C. cyanus* resistance was found mainly against acetolactate synthase (ALS) inhibitors, but no ALS sequence information concerning the herbicide resistance mechanism has been published yet. The aim of this study was to determine the *ALS* sequences for biotypes susceptible and resistant to tribenuron-methyl in order to identify mutations that may be associated with the resistance emergence. DNA isolation from susceptible and resistant plants was followed by PCR amplification and *ALS* sequencing. As a result, different lengths of DNA products were obtained. Moreover, both nucleotide and amino acid sequence analysis revealed high sequence variability within one plant as well as between plants from the same biotype. In a few resistant plants, four changes in the amino acid sequence were identified in comparison to those in the susceptible ones. However, these preliminary studies require further investigation toward confirming the significance of these mutations in herbicide resistance development. This study provides preliminary information contributing to the research on the *C. cyanus* target-site resistance mechanism.

Keywords: *Centaurea cyanus*; herbicide resistance; ALS sequence; acetolactate synthase; cornflower; genetic variability

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1. Introduction

Centaurea cyanus L. is an annual weed species belonging to the Asteraceae family. It is commonly found in fields with cereals, sugar beet, and maize. In certain countries, it is increasingly rare, while in others, e.g., in those in Central and Eastern Europe, it is more frequently encountered. Currently, 22 active ingredients of herbicides are recommended for controlling *C. cyanus* in Europe [1]. The herbicides that are recommended for the control of this weed belong mainly to acetolactate synthase (ALS) and photosystem II inhibitors and synthetic auxins groups. However, frequent use of herbicides with the same mechanism of action leads to the emergence of weed resistance. The first reports concerning *C. cyanus* resistance to chlorsulfuron (ALS inhibitor) date back to 2008 [2] and its cross-resistance to chlorosulfuron and tribenuron-methyl was detected in 2010 [3]. Thus far, resistant *C. cyanus* biotypes have been identified only in Poland [4].

Herbicide resistance of weeds poses a threat to agricultural production; therefore, identification of the causes of the emergence of this phenomenon is of great economic significance and necessary to establish effective weed management procedures. The mechanism of the herbicide resistance emergence in *C. cyanus* plants is yet unknown. One of the approaches aiming at explaining the target-site resistance (TSR) mechanism is the analysis

of the sequences of the herbicide target enzymes. Early studies on *C. cyanus* resistance towards ALS inhibitors suggested both TSR and non-target-site resistance (NTSR) mechanisms [5]. To date, mutations at eight positions within ALS amino acid sequence (A122, P197, A205, D376, R376, W574, S653, and D654) have been confirmed as responsible for herbicide resistance in numerous weed species [4]. Mutations at P197 and W574 are the most commonly identified, where P197 is associated mostly with the sulfonylurea herbicide chemical group, while W574 is mostly selected by sulfonylurea and imidazolinone groups [6].

Thus far, there is a lack of information concerning the nucleotide sequences of herbicide target enzyme genes of herbicide-susceptible and -resistant *C. cyanus* plants in gene repository databases. Only one *ALS* sequence (accession number: MK941142) was deposited in GenBank; however, no resistance status of the source plant was provided. Therefore, the aim of this study was to analyse and compare the *ALS* sequences of *C. cyanus* plants that belong to the biotypes susceptible and resistant to tribenuron-methyl.

2. Materials and Methods

Biotypes of *C. cyanus* that were found to be susceptible (2 biotypes) and resistant (3 biotypes) to ALS inhibitor tribenuron-methyl, obtained from different locations in Poland, were selected for analysis. Their susceptibility to herbicide treatment was assessed by the determination of ED_{50} (effective dose of active ingredient (ai) causing a 50% reduction in plant biomass). For this purpose, the seeds were sown in pots placed under controlled conditions in the greenhouse. Plants at the 12–13th growth stage (according to BBCH scale) were treated with Lumer 50 WG (ai tribenuron-methyl 500 g kg^{-1}, ADAMA Agriculture B.V., Schaffhausen, Switzerland) at specific doses: for resistant populations, 0N, 0.5N, 1N, 2N, 4N, 8N, 16N, 32N, and for susceptible populations, 0N, 1/16N, 1/8N, 1/4N, 1/2N, 1N, 2N, 4N, where N is the maximal recommended dose of the herbicide (30 g ha^{-1}, i.e., 15 g ha^{-1} of active substance). Twenty-one days after herbicide application, the plants were harvested and their biomasses were measured at the population level. Leaves from four plants from the resistant biotypes treated with a 1N dose of the herbicide and from the untreated plants from the susceptible biotypes were harvested for molecular analyses.

The leaves were ground in a mortar using liquid nitrogen. Genomic DNA was isolated using the NucleoSpin Plant II Mini Kit for DNA from plants (Mecherey-Nagel, Düren, Germany). To amplify the *C. cyanus ALS* sequence, a pair of degenerated primers was designed, based on the alignment of the *ALS* coding sequences of plant species belonging to the Asteraceae family, deposited in GenBank. The alignment was performed using BioEdit Sequence Alignment Editor (version 7.5.5) [7]. PCR was carried out in 50 µL of the reaction mixture containing 1X Q5 Reaction Buffer (NEB, Ipswich, MA, USA), 200 µM dNTPs, 0.5 µM forward primer (5′ CGTKCTBGTRGAAGCCYTSGA 3′), 0.5 µM reverse primer (5′ TCAATATTKYGTTCTKCCATCDCC 3′), 200 ng of genomic DNA, and 1 U of Q5 High-Fidelity DNA Polymerase (NEB). PCR was performed in a Mastercycler nexus (Eppendorf, Hamburg, Germany) with an initial denaturation at 98 °C for 30 s, followed by 35 cycles of amplification: 10 s at 98 °C, 30 s at a 63 °C, and 1 min at 72 °C, with a final step of 2 min at 72 °C. The reaction products were separated with 1% gel electrophoresis, purified from the gel with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), ligated to pJET1.2 plasmid using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA), and cloned into DH10B *Escherichia coli* competent cells. The colony PCR method was used for positive colony verification. The plasmids containing the inserted *ALS* sequence were isolated from *E. coli* cells using NucleoSpin Plasmid (Mecherey-Nagel). The presence of the insert in plasmids was confirmed by digestion with *BglII*. Three plasmids were sequenced per plant. DNA inserts were sequenced by Genomed (Warsaw, Poland). Sequencing data were analysed using the BioEdit Sequence Alignment Editor 7.5.5 [7] and visualised by GeneDoc (version 2.7.000) [8].

3. Results and Discussion

The level of sensitivity to the herbicide of the plants belonging to two biotypes of *C. cyanus* susceptible to the ALS inhibitor (tribenuron-methyl) and three plants resistant to it were analysed and compared. These biotypes were composed of 100% sensitive or fully resistant individual plants. Their susceptibility to the herbicide was assessed by the determination of ED_{50}. The effective dose of tribenuron-methyl causing 50% loss in plant biomass was measured at the population level. According to the results, low doses of the herbicide, such as 4.73 g h^{-1} and 6.07 g h^{-1}, were necessary for S1 and S2 biotypes' biomass reduction, respectively (Table 1). The application of the 16N dose of the herbicide to all plants of the resistant biotypes was insufficient for ED_{50} determination. For these plants, the dose of 16N did not cause visible signs of contact with the herbicide (Table 1).

Table 1. The effective doses (ED_{50}) of tribenuron-methyl causing 50% loss in plant biomass measured for *Centaurea cyanus* biotypes susceptible and resistant to the herbicide studied.

Biotype	Biotype Number	ED_{50} (g h^{-1})
Susceptible	1	4.73
	2	6.07
Resistant	1	>480
	2	>480
	3	>480

ALS sequence amplification resulted in the generation of 1699 to 1708 bp fragments that encompassed amino acids from 109 to 663 of the ALS sequence (according to the *Arabidopsis thaliana* amino acid numbering in ALS sequence, accession number: P17597). The involvement of P197 mutation in TSR against sulfonylurea herbicides in *C. cyanus* has been previously indicated; however, no more detailed results were presented [9]. The amplified sequences were sufficient to screen for the presence of mutations that were found to be involved in resistance development to ALS inhibitors in other weed species (A122, P197, A205, D376, R377, W574, S653, and G654) [4].

Two biotypes susceptible to tribenuron-methyl and three resistant to it were subjected to analysis. More precisely, four plants from each biotype and six plasmids from each plant were analysed. Nucleotide sequence analysis revealed high variability between the obtained sequences. The analysed fragments were also found to differ in length, which was the effect of the presence of three-nucleotide indels in the sequence fragments located between ALS functional regions. Moreover, changes in the nucleotides at multiple positions within the analysed fragments were observed. Overall, out of the 120 nucleotide sequences obtained from all plasmids, there were 42 different sequence variants, including eight sequences that were found in plasmids obtained from both susceptible and resistant plants. Of these 42 different sequence variants, eight were unique to susceptible plants, whereas, 26 were unique to resistant ones. In total, synonymous changes were found at 142 positions within the nucleotide sequences, whereas, at 65 positions, nucleotide changes resulted in the incorporation of other amino acids. The majority of differences in the amino acid sequences were found in both susceptible and resistant biotypes, which indicates that their significance in herbicide resistance emergence may not be vital. However, four mutations (L179I, N404R, I468V, and V525I) that were located in the functional regions of ALS were found only in the resistant plants (Figures 1–3). All these amino acid changes were detected in at least two plasmids (out of six) from individual plants resistant to the herbicide, while mutations N404R and V525I were found in one out of three plasmids in four resistant plants, which implies their heterozygosity. Additionally, these two changes were identified in the same plasmids simultaneously. No earlier detected mutations related to herbicide resistance in other weed species were found [4,6]. The presence of P197 mutation, suggested to be present in one resistant biotype in Poland [9], was not confirmed. P197 mutation is

one of the most commonly identified amino acid substitutions and, together with A205, is considered to confer sulfonylurea-specific resistance [6]. N404R and V525I mutations constitute novel changes within the ALS amino acid sequence in biotypes resistant to ALS inhibitors; therefore, more detailed studies involving numerous samples should be carried out. The analysed nucleotide sequences were deposited in GenBank under accession numbers MZ561651-MZ561687 and OL332826-OL332835.

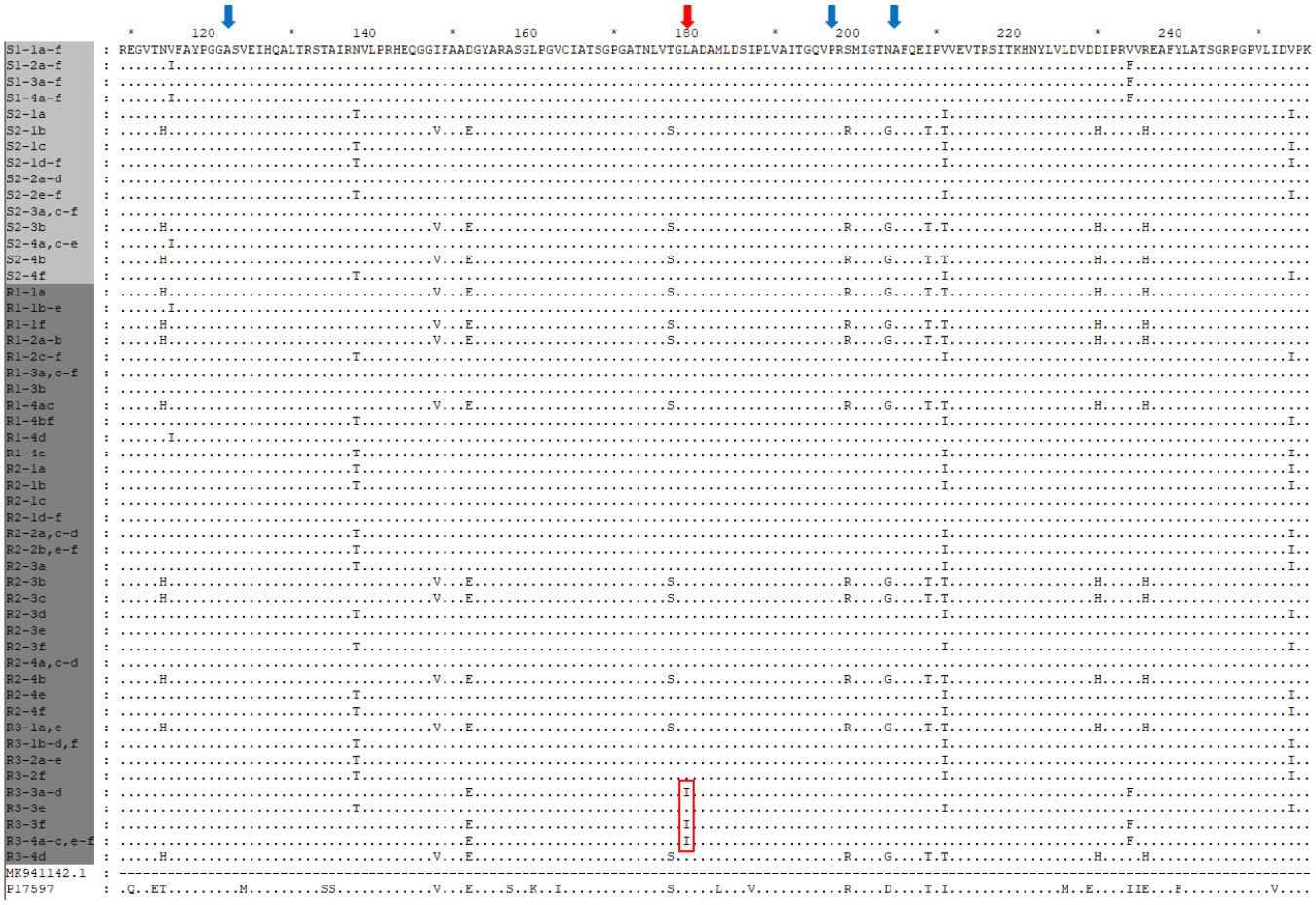

Figure 1. The alignment of *Centaurea cyanus* ALS amino acid sequences encompassing pyrimidine (PYR) binding domain of pyruvate oxidase (POX) and related protein regions. The sequences were derived from the plants susceptible (S) and resistant (R) to Lumer 50 WG. The numbers in the sequence names represent the plant number; a–f—clone name; MK941142—*C. cyanus ALS* sequence GenBank accession number (the nucleotide sequence was translated to amino acid sequence); P17597—*Arabidopsis thaliana* ALS amino acid sequence GenBank accession number. The red frames and red arrows indicate amino acid mutations found in *C. cyanus* R plants. The blue arrows indicate the positions of mutations known for conferring resistance to ALS-inhibiting herbicides at positions A122, P197, and A205, respectively. The amino acid numbering refers to *A. thaliana* ALS sequence.

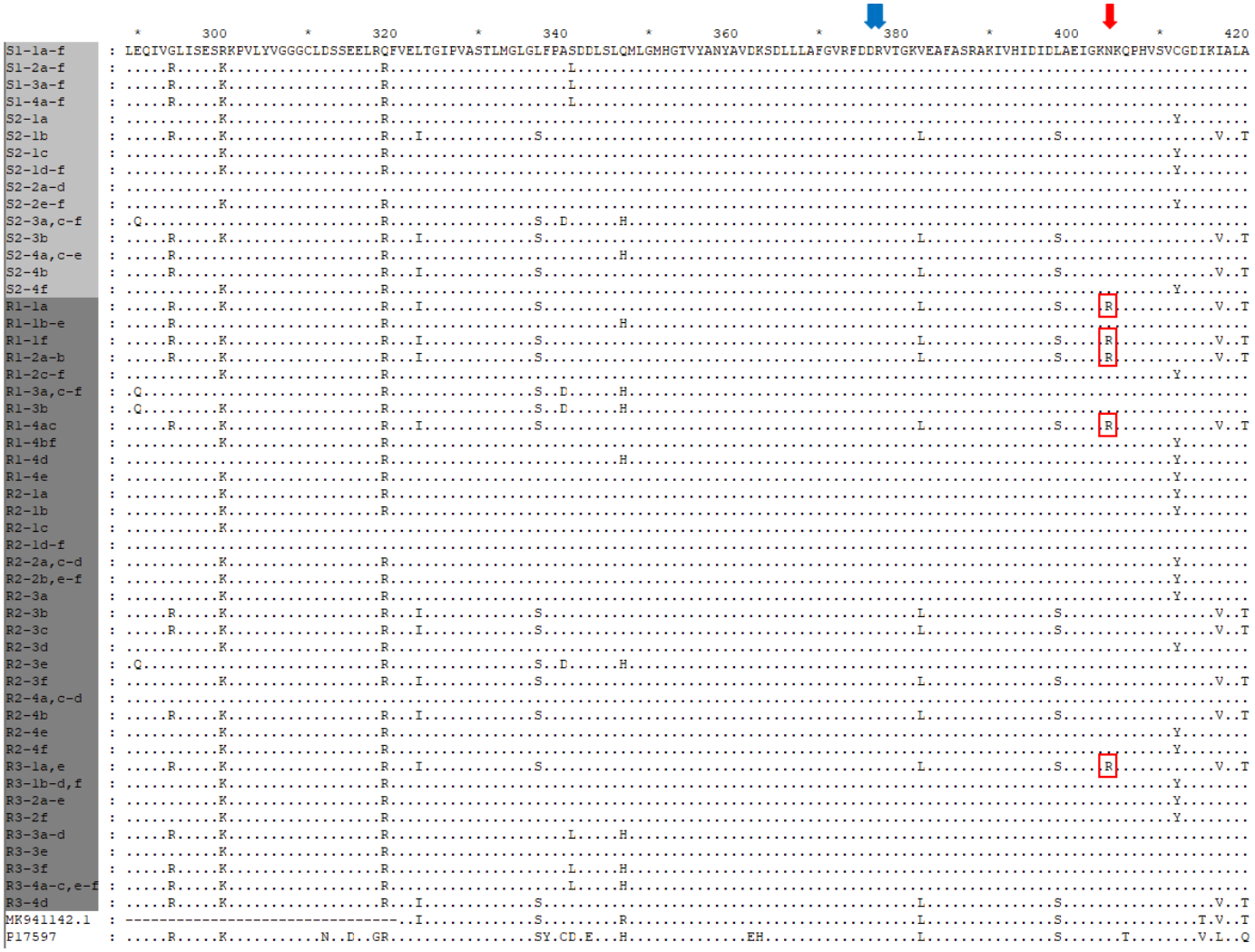

Figure 2. The alignment of *Centaurea cyanus* ALS amino acid sequences encompassing thiamine pyrophosphate enzyme, central domain. The sequences were derived from the plants susceptible (S) and resistant (R) to Lumer 50 WG. The numbers in sequence names represent the plant number; a–f—clone name; MK941142—*C. cyanus ALS* sequence GenBank accession number (the nucleotide sequence was translated to amino acid sequence); P17597—*Arabidopsis thaliana* ALS amino acid sequence GenBank accession number. The red frames and red arrows indicate amino acid mutations found in *C. cyanus* R plants. The blue arrows indicate the positions of mutations known for conferring resistance to ALS-inhibiting herbicides at positions D376 and R377, respectively. The amino acid numbering refers to *A. thaliana* ALS sequence.

For four plants belonging to one susceptible biotype, all six sequences (obtained from six plasmids from the same plant) were the same, but within the same biotype, the sequences derived from different plants significantly differed. In some cases, the sequencing resulted in the identification of six divergent sequences from one plant. Such a high number of polymorphisms in the ALS nucleotide sequence was also observed in other Asteraceae family species, namely in *Ambrosia artemisiifolia* L. [10,11], as well as in other plant families and species, such as *Alopecurus aequalis* [12] or *Zea mays* [13]. The reason for such differences in the obtained sequences may be the copy number variation. Multiple gene copies may increase the effective gene dosage, which may influence the phenotype [14]. This mechanism has been described in the context of the evolution of *Amaranthus palmeri* resistance to glyphosate. It was revealed that *A. palmeri* resistance to glyphosate was driven by the elevated 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene copy number, followed by increased EPSPS transcript and protein levels, along with enhanced glyphosate dose survival rate [15]. In the case of *C. cyanus*, high variability between *ALS* sequences was observed in biotypes both susceptible and resistant

to tribenuron-methyl; therefore, the possibility of the involvement of the copy number variation in resistance emergence cannot be excluded, but it also cannot be confirmed. This phenomenon can be explained by the natural variability of *ALS*. The high genetic variability of *C. cyanus* plants was confirmed in the analysis of ten microsatellite markers showing high polymorphism [16]. Another study concerning the analysis of leaf isozyme markers also highlighted the high genetic diversity of *C. cyanus* populations [17]. It should be noted that despite low levels of genetic differentiation between populations, fine-scale spatial genetic structure was observed within populations [16].

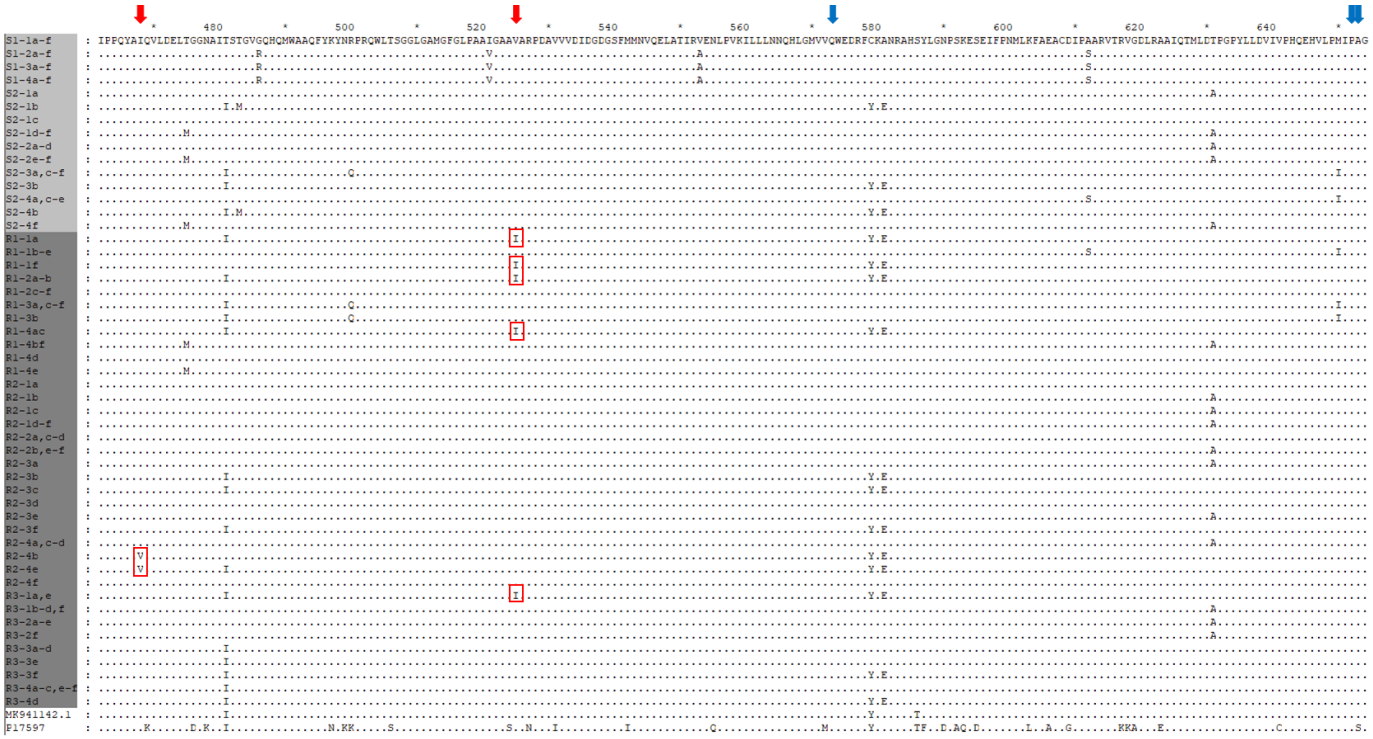

Figure 3. The alignment of *Centaurea cyanus* ALS amino acid sequences encompassing thiamine pyrophosphate binding domain. The sequences were derived from the plants susceptible (S) and resistant (R) to Lumer 50 WG. The numbers in sequence names represent the plant number; a–f—clone name; MK941142—*C. cyanus ALS* sequence GenBank accession number (the nucleotide sequence was translated to amino acid sequence); P17597—*Arabidopsis thaliana* ALS amino acid sequence GenBank accession number. The red frames and red arrows indicate amino acid mutations found in *C. cyanus* R plants. The blue arrows indicate the positions of mutations known for conferring resistance to ALS-inhibiting herbicides at positions W574, A653, and G654, respectively. The amino acid numbering refers to *A. thaliana* ALS sequence.

To sum up, our study revealed high variability in the obtained ALS nucleotide, as well as amino acid sequences within and between the analysed plants. Multiple changes were observed in the biotypes both susceptible and resistant to tribenuron-methyl; therefore, this phenomenon can be treated as the natural variability in this species. A few mutations were found only in the resistant plants, among which N404R and V525I were observed in four plants, but no mutations previously associated with conferring the resistance to ALS inhibitors were observed. Currently, the connection between the found mutations and their contribution to herbicide resistance emergence is difficult to prove and thus further analyses are required in order to confirm the presence of these mutations in a greater number of biotypes and plants. The observed mutations do not provide a strong basis for the target-site resistance mechanism in *C. cyanus* plants resistant to tribenuron-methyl. Therefore, it can be assumed that the resistance to the herbicide may result from the non-target-site mechanism as well, and this issue requires more detailed further studies.

Owing to the fact that there is scarce information about the *ALS* sequence of the herbicide-susceptible and -resistant *C. cyanus* biotypes, these studies provide the first

comparative analysis between the herbicide-susceptible and -resistant *C. cyanus* plants, which may be useful for future studies concerning herbicide resistance in this species. Moreover, our work also confirms the high genetic variability in this species.

Author Contributions: Designed the experiment and methodology of the analysis, B.W.; provided samples of weed populations, T.P.; performed the study and analysed the results, B.W.; wrote the manuscript, B.W.; analysed sequence data, B.W.; revised the manuscript, B.W. and T.P. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The fragments of *Centaurea cyanus* acetolactate synthase gene sequences are available in the GenBank database under accession numbers MZ561651-MZ561687 and OL332826-OL332835.

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