Differences in Amino Acid Composition of Carrot \(\alpha\)-Tubulin Potentially Confer the Resistance to Dinitroaniline Herbicides

**Aim.** To reveal the features of amino acid composition of carrot \(\alpha\)-tubulin isotypes that potentially determine natural tolerance to dinitroaniline herbicides. **Methods.** Literature and database search. Comparison of protein sequences and structures: multiple sequence alignment, phylogenetic profiling, protein and ligand structure modeling, etc. **Results.** Genomic and proteomic analysis of *Daucus carota* has revealed at least eight unique isotypes of \(\alpha\)-tubulin that differ in amino acid sequences and gene loci. Remarkable differences in amino acid composition of the dinitroaniline-binding-like (DBL) region of analyzed \(\alpha\)-tubulin have been revealed, which may be the reason of its natural resistance to these compounds. **Conclusions.** Differences in amino acids at positions of canonical mutations – Cys4 (TBA1, 2, 3, 6, 7 and 8), Thr53 (TBA6), Ile202 (TBA1 and 7) and Met202 (TBA5), as well as previously undescribed non-canonical substitutions – Ile4 (TBA4 and 5), Cys52 (TBA6), Ser201 (TBA1, 2, 3 and 8) and Val194 (TBA4 and 5), were noted as potentially associated with natural tolerance of the carrot to dinitroaniline herbicides.

**Keywords:** \(\alpha\)-tubulin, dinitroaniline, resistance, oryzalin, microtubules, herbicides.

Carrot is a remarkable example of natural tolerance to dinitroaniline herbicides (www.weedscience.org). For the first time, its resistance was reported by Vaughan and Vaughn in 1988, when immunofluorescent and electron microscopy indicated that microtubules of the carrot were unaffected by dinitroanilines [1]. Treatment with trifluralin as well as other dinitroaniline herbicides resulted in no apparent alteration of normal interphase cell structure or mitotic spindle microtubules. Likewise, none of the other ultrastructural changes associated with dinitroaniline herbicide treatment (multiple nuclei, arrested mitosis, lobed nuclei, abnormal cell wall formation) were noted. In addition, carrot is insensitive to similar compounds – hexanitrodiphenylamine and amiprophosmethyl [2]. It is known that \(\alpha\)-tubulin is the confirmed molecular target of dinitroaniline compounds, and it has been hypothesized that carrot resistance might depend on amino acid composition in the ligand-binding site. Currently, there are numerous examples of tubulin mutations leading to dinitroaniline resistance in algae, fungi, mammalian and toxoplasma [3–14, 16, 17, 20]. However, the resistance exhibited by carrot, appears to be a case of 'pre-existing' resistance in this species [1].

In the present study, we aimed to analyze amino acid composition of the target site in carrot \(\alpha\)-tubulin isotypes, and to reveal whatever this native resistance is caused by amino acid substitution(s) in the site. It should be noted that there are several hypotheses of the protein-ligand interaction between \(\alpha\)-tubulin and dinitroaniline compounds [7, 13, 14, 18, 19–22]. In our study, we adhered to recent hypothesis proposed by Aguayo-Ortiz et al. (2022) using the model of \(\alpha\)-tubulin from *T. gondii* [18]. In our opinion, this hypothesis is not only the most structurally substantiated, but also overlaps with the majority of known mutations, associated with dinitroaniline resistance [8, 20]. Since this hypothesis is based on the ligand-induced mechanism of site formation, our study involved the analysis of the target binding site in open and closed conformations [18].

**Materials and methods**

Complete amino acid sequences of tubulin isotypes were obtained from the UniProtKB (www.uniprot.org). RCSB Protein Data Bank (www.rcsb.org) was used for structural information and templates for structural modeling. Sequences alignments were made in ClustalX v. 2.1 (www.clustal.org).

The information from GenBank (www.ncbi.nlm.nih.gov/genbank/), USDA ARS Carrot Genome Project and data from European
Nucleotid Archive (https://plants.ensembl.org) was the initial points for our genomic analysis. Primary ranking was carried out according to the neighbor-joining (NJ) algorithm and additional verification was performed using BLAST (blastp, tblastn and tblastx) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Visualization and analysis of the NJ-tree were performed in MEGA-X (www.megasoftware.net). The gene loci information (chromosome, nucleotide coordinates and ORF direction) associated with deposed a. a. products, were the key argument in evaluation of unique tubulin isotypes.

Analyzing the Dinitroaniline-Binding-Like (DBL) region, we operate the model of dinitroaniline binding, proposed by the R. Aguayo-Ortiz, L. Dominiguez, 2022 for Toxoplasma gondii [18]. Due to the ligand-induced mechanism of site formation, it was necessary to model all carrot α-tubulins in open and closed conformations. The closed form was built using AlphaFold2 (https://alphafold.ebi.ac.uk), while an open conformation was constructed using Swiss-Model server (https://swissmodel.expasy.org) based on structures of previously built homological complexes. Also, open conformations of α-tubulins were compared with RCSB Protein Data Bank structure 5fnv (DOI: 10.2210/pdb5FNV/pdb) of Gallus gallus (α-tubulin-Pironetin complex), used R. Aguayo-Ortiz, L. Dominiguez, 2022 for dinitroaniline site prediction in T. gondii [18].

All structures were optimized in the GROMACS (www.gromacs.org) with Charmm36ff (www.charmm.org) in physiological model of solvent. Amino acids, forming active site region were specified in PyMOL, based on 6 Å distance from reference ligands (oryzalin, trifluralin) transferred from previously constructed complexes.

Alignment of structures, site specification, description and visualization were processed in PyMOL V.2.5.4 (Schrödinger, LLC – www.pymol.org).


Results and discussion
Primary sequence sampling was performed based on UniProtKB and GeneBank blastp scans against a single annotated Daucus carota α-tubulin (Q9FT36, TBA_DAUC). Additionally, other annotated plant α-tubulins have been used. A direct scan of the D. carota genome was also performed using translational algorithms blastx and tblastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primary combined sample of 59 sequences were aligned in ClustalX and clustered using the neighbor-joining (-NJ) method (www.clustal.org). The final verification of the uniqueness of the amino acid sequence hypotheses was performed by matching gene loci using the Genome Data Viewer (https://www.ncbi.nlm.nih.gov/books/NBK21101/) and was performed using the ASM162521v1 genomic assembly (United States Department of Agriculture (USDA - www.usda.gov)).

In total, eight sequences of α-tubulins were indentified within the genome of D. carota (Table 1). Since the annotation of these isotypes is still in progress, the corresponding amino acid sequences are deposited in UniProtKB under primary numbers. For convenience, we referred these proteins as TBA1 – TBA8 in the order of their occurrence in the genome – from chromosome 1 to 7.

To determine amino acids, topologically located in the DBL region, we performed structural alignment of 3D-models of carrot α-tubulin isotypes (in closed and open conformations) and previously built reference (control) complexes of Hordeum vulgare α-tubulin (TBA3_HORVU) in complex with oryzalin and trifluralin. In total, we used 10 reference complexes with each ligand, which were retrieved regularly from the frames of the stable regions of molecular dynamics (Gromacs, >90 ns). Due to differences of closed and open conformations of the target site, the protocol of template modeling varied (see Materials and methods). In order to minimize local tensions in 3D-models and to reach more natural conformational state, all D. carota α-tubulin models were optimized in Gromacs (Charmm36 ff). Structural alignment of reference complexes and 8 α-tubulin isotypes of D. carota was performed in PyMOL. The RMSD values of all aligned structures were <1, indicating a significant similarity of all 3D-models of α-tubulin from D. carota and H. vulgare. The structures of ligands from the reference complexes
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were transferred without changing of 3D-coordinates into a separate object and grouped with 3D-models of carrot α-tubulin. This allowed us to select amino acids forming the potential site in open and closed conformations, with a distance of 6 Å from the cluster of reference ligands (Fig. 1).

More than 40 α-tubulin amino acid substitutions are currently known to be associated with dinitroaniline resistance. [3–14, 16, 17, 20] Features of the amino acid composition of the target site is one of the most probable reasons for D. carota resistance to dinitroanilines. In total, we have found 41 documented a. a. substitutions (in α-tubulin) causing the resistance to dinitroanilines. This information was used as the initial point for search for similar substitutions among amino acids of a hypothetical "site" in carrot α-tubulin iso-
types. (Fig. 1 A, B and Fig. 2).

At 6 Å distance from the reference ligands cluster, selected amino acids formed ten polypeptide fragments (I-X). For convenience of subsequent search, annotation of perspective positions were performed in relation to these fragments. Thus, fragment I at position 4 overlaps with previously described mutation Val4Leu [13]. In this position, carrot isotypes TBA1, TBA2, TBA3, TBA6, TBA7, and TBA8 had a polar uncharged cysteine instead of a canonical nonpolar aliphatic amino acid. In addition, TBA4 and TBA5 isotypes instead canonical valine (Val4) possess isoleucine (Ile4). At the same time, the conservatism of the adjacent Ser6 and His8 was retained.

Table 1. Eight α-tubulin isotypes identified in Daucus carota L.

<table>
<thead>
<tr>
<th>n.n.</th>
<th>Name</th>
<th>UniProtKB</th>
<th>Gene Locus Coordinates</th>
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<tbody>
<tr>
<td>1</td>
<td>TBA1</td>
<td>A0A162B2L8</td>
<td>Chr1: 21,717,891 – 21,720,672 (+)</td>
</tr>
<tr>
<td>2</td>
<td>TBA2 / TBA</td>
<td>Q9FT36 / A0A3G2K878</td>
<td>Chr3: 28,398,089 – 28,401,001 (+)</td>
</tr>
<tr>
<td>3</td>
<td>TBA3</td>
<td>A0A162AKE6</td>
<td>Chr3: 32,877,823 – 32,880,324 (-)</td>
</tr>
<tr>
<td>4</td>
<td>TBA4</td>
<td>A0A165YBU4</td>
<td>Chr4: 28,288,081 – 28,292,957 (+)</td>
</tr>
<tr>
<td>5</td>
<td>TBA5</td>
<td>A0A164Y0E4</td>
<td>Chr5: 10,965,871 – 10,968,026 (+)</td>
</tr>
<tr>
<td>6</td>
<td>TBA6</td>
<td>A0A161XE0</td>
<td>Chr6: 22,157,899 – 22,160,392 (+)</td>
</tr>
<tr>
<td>7</td>
<td>TBA7</td>
<td>A0A164VQB6</td>
<td>Chr6: 18,218,088 – 18,221,142 (-)</td>
</tr>
<tr>
<td>8</td>
<td>TBA8</td>
<td>A0A164U182</td>
<td>Chr7: 26,572,720 – 26,574,713 (+)</td>
</tr>
</tbody>
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Fig. 1. Ribbon diagram (A) of the hypothetical Dinitroaniline Binding-Like (DBL) region and the group of control conformations of oryzalin and trifluralin (10 per compound) used to determine the amino acid environment of the target site in isotypes of carrot α-tubulin with a distance of 6Å from the reference cluster of ligands (B). Orange – open conformation of the site; Cyan – closed conformation of the site.
Fragment II covers the site of previously described Phe52 mutations (Phe52Ile, Phe52Leu, Phe52Tyr) [14, 20]. In this position, we have found a substitution of aromatic Phe52 for polar and uncharged Thr53 (Phe53Thr). However, this substitution was the only one in the TBA6. Noteworthy, this isotype had cysteine substitution instead of canonical Thr52.

Fragment III overlaps with the site of Leu136Phe mutation, previously described in A. aequalis [10], S. viridis [8], T. thermophila [12], T. gondii [20]. However, in carrot, this region remains conservative, relatively to dinitroaniline-sensitive type (DST) of α-tubulin. A similar pattern was also observed in the case of Fragment IV. It retains the conservatism of the dinitroaniline-sensitive type, but overlaps with the site of mutations previously described in T. gondii (Ser165Ala, Ser165Pro and Ser165Thr) [20].

Fragment V overlaps with the site of mutation Val202Phe, previously described for α-tubulin in A. aequalis [10] and L. rigidum [6]. In the carrot α-tubulin isotypes, the position of Val202 is conserved in DST manner for TBA2, TBA3, TBA4, TBA6, and TBA8. In the isotypes TBA1 and TBA7, Val202 is replaced by isoleucine (Ile202), and in the isotype TBA5 by methionine (Met202). In our opinion, more remarkable for this fragment is the previously undescribed substitution of canonical Ala201 for polar serine (Ser201), which was found in isotypes TBA1, TBA2, TBA3, and TBA8. In addition, in isotypes TBA4 and TBA5, at this position we have found presence of valine (Val201). Although, as the analysis of DST α-tubulins has demonstrated, the Ala/Val201 variation is standard and it is unlikely associated with the dinitroaniline resistance.

Fragment VI was the largest, and overlapped with a number of sites of known mutations: Ile235Leu/Thr/Val [13, 20], Leu238Val [20], Thr239Ile [6, 8, 17, 20], Arg243Cys/Lys/Met/Ser [7, 20] and Val252Leu [12, 20]. However, in carrot, all of the positions mentioned above correspond to dinitroaniline-sensitive type.

Fragment VII overlaps with position of known mutation Met268Thr, but in all carrot α-tubulin isotypes it retains dinitroaniline-sensitive type of amino acid composition. The remaining Fragments XIII, IX and X retained canonical DST conservatism and do not coincide with the sites of known dinitroaniline-resistant mutations.

**Conclusions**

Firstly, the revision of current genomic and proteomic information, revealed that *Daucus carota* has at least 8 α-tubulin isotypes, which demonstrate unique amino acid sequences and encoded by distinct gene loci. Secondly, the dinitroaniline binding-like region in α-tubulin isotypes of *D. carota* contain amino acid residues, which may be related to carrot’s natural dinitroaniline resistance. Amino acids in the canonical positions – Cys4 (TBA 1, 2, 3, 6, 7 and 8), Thr53 (TBA 6), Ile202 (TBA 1 and 7), Met202 (TBA 5), as well as positions, were identified in the present study – Ile4 (TBA4 and 5), Cys52 (TBA 6), Ser201 (TBA 1, 2, 3 and 8) and Val194 (TBA 4 and 5) were selected as potential causes preventing dinitroanilines interaction with α-tubulins in *D. carota* and will be the objects of further bioinformatical and laboratory studies.

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МЕЛЬНИК О. Г., БЛЮМ Р. Я., КАРПОВ П. А.
Державна установа «Інститут харчової біотехнології та геноміки НАН України», Україна, 04123, м. Київ, вул. Байди-Вишневецького, 2А

ВІДМІННОСТІ АМІНОКИСЛОТНОГО СКЛАДУ α-ТУБУЛІНІВ DAUCUS CAROTA, ЯКІ ПОТЕНЦІЙНО ОБУМОВЛЮЮТЬ СТІЙКІСТЬ ДО ГЕРБІЦІДІВ ДИНІТРОАНІЛІНОВОГО РЯДУ

**Мета.** Виявити особливості амінокислотного складу ізотипів α-тубуліну Daucus carota, котрі могли б обумовлювати природну стійкість до дії гербіцидів динітроанілінового ряду. **Методи.** Аналіз баз даних та літературних джерел. Вирівняння послідовностей і філогенетичний аналіз. Порівняльний аналіз структур білків, лігандів та їх комплексів. **Результати.** Геномний і протеомний аналіз D. carota виявили щонайменше 8 унікальних ізотипів α-тубуліну, які відрізняються від амінокислотними послідовностями, а також, локусами генів. Амінокислотний склад у ділянці, котра топологічно відповідає сайту зв’язування динітроанілінів (DBL) виявив у досліджених α-тубулінів відмінності, які потенційно обумовлюють природну стійкість D. carota до цих сполук. **Висновки.** Знайдено відмінності канонічних амінокислот у положеннях, що відповідають відомим мутаціям – Cys4 (TBA 1, 2, 3, 6, 7 і 8), Thr53 (TBA 6), Ile202 (TBA 1 і 7) і Met202 (TBA 5), а також неописани ранише неканонічні заміни – Ile4 (TBA 4 і 5), Cys52 (TBA 6), Ser201 і Val194 (TBA 4 і 5), що були відібрані як ті, що потенційно обумовлюють природну толерантність D. carota до гербіцидів динітроанілінового ряду.

**Ключові слова:** α-тубулін, динітроанілін, резистентність, орізаль, мікротрубочки, гербіциди.