Research Article/Araştırma Makalesi

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MEDITERRANEAN AGRICULTURAL SCIENCES (2019) 32(2): 201-210
DOI: 10.29136/mediterranean.559688

Insights into herbicide resistance: Bioinformatics analyses of *AHAS* (acetohydroxyacid synthase) genes in tomato and potato

Herbisit dayanıklılığını anlamak: Domates ve patatesteki AHAS (asetohidroksiasit sentetaz) genlerinin biyoinformatik analizleri

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ARTICLE INFO

Received 01 May 2019
Received in revised form 05 July 2019
Accepted 11 July 2019

***Keywords*:**

Acetohydroxyacid synthase
Acetolactate synthase
Herbicide resistance
Mutation

ABSTRACT

The identification of enzymes' mutable sites is important to the development of herbicide resistant crops and for weed control practices. The objective of this study was to provide insights into mutable residues causing resistance to the acetohydroxyacid synthase enzyme (AHAS, EC 2.2.1.6) inhibitor herbicides in the tomato (SlAHAS) and potato (StAHAS) through bioinformatics approaches. The results showed AHAS proteins investigated in this study were highly conserved but differed in length. Mutation analyses showed that Lys541 and Val542 in SlAHAS were mutable sites for preservation of the enzyme activity. While Ala, Phe, Arg, and Val residues were found to be substitutable with Lys541, Ile was exchangeable for Val542. Similarly, Ile124, Met266, and Leu272 in StAHAS were identified as protein stabilizing residues. In this respect, Lys and Arg were substitutable residues for Ile124, whereas Leu was for Met266 and Ala, Pro and Ser were suitable residues for Leu272 regarding enzyme stabilization. The docking analyses displayed that the best binding affinities were obtained for Ser387, Arg235, and His341 for chlorosulfuron (CS) and Phe11, Ala40, and His341 have the highest binding score for imazaquin (IQ) in SlAHAS. As for StAHAS, Lys232, Asn123, and Arg53 residues were found to bind with CS whereas Lys405, Lys489, and Arg268 amino acids were identified as sites where IQ bound. His341 and Gln478 were binding residues for both CS and IQ in SlAHAS whereas both ligands were found to bind with Val61 and Arg366 in StAHAS. Arg366 was identified as a binding site in SlAHAS for IQ as well.

MAKALE BİLGİSİ

Alınış tarihi 01 Mayıs 2018
Düzeltilme tarihi 05 Temmuz 2019
Kabul tarihi 11 Temmuz 2019

***Anahtar Kelimeler*:**

Asetohidroksiasit sentetaz
Asetolaktat sentetaz
Herbisit dayanıklılığı
Mutasyon

ÖZ

Enzimlerin mutasyon bölgelerinin belirlenmesi herbisitlere dayanıklı bitkilerin yetiştirilmesi ve yabancı ot kontrol uygulamalarının başarısı için önemlidir. Bu çalışma domates (SlAHAS) ve patatesteki (StAHAS) asetohidroksiasit sentetaz (AHAS, EC 2.2.1.6) enzimlerine herbisit dayanıklılığını sağlayacak mutasyon bölgelerinin biyoinformatik yöntemlerle belirlenmesi amacıyla yapılmıştır. AHAS proteinleri evrimsel olarak yüksek oranda korunmasına rağmen bu proteinlerin uzunlukları farklılık göstermektedir. SlAHAS'ta Lys541 ve Val542 amino asitleri (aa) enzim aktivitesi için önem taşımaktadır ve Lys541 Ala, Phe, Arg, ve Val aa ile yer değiştirebilirken; Ile sadece Val542 ile yer değiştirebilir aa olarak bulunmuştur. Benzer şekilde StAHAS'ta Ile124, Met266 ve Leu272 stabilizasyonu sağlayacı aa olarak bulunmuştur. Lys ve Arg, Ile124 ile değişebilir aa olarak saptanırken; Leu, Met266 ile ve Ala, Pro ve Ser ise Leu272 ile enzim stabilizayonunu sağlayıcı yer değiştirebilir aa olarak bulunmuştur. SlAHAS'taki kenetlenme analizlerine göre klorosülfüron (CS) için Ser387, Arg235 ve His341; imazakin (IQ) içinse Phe11, Ala40 ve His341 en yüksek bağlanma sonuçlarını vermiştir. StAHAS'ta ise Lys232, Asn123 ve Arg53'ün CS ile bağlandığı; Lys405, Lys489 ve Arg268 ise IQ ile bağlanabilecek aa'ler olduğu tespit edilmiştir. His341 ve Gln478'in CS ve IQ ile SlAHAS'ta; Val61 ve Arg366'nın ise StAHAS'ta sırasıyla her iki ligand ile bağ yapabildiği görülmüştür. Bunun yanısıra Arg366 SlAHAS'ta IQ ligantının bağlanabileceği aa olarak bulunmuştur.

1. Introduction

Weed control in crop cultivation has been achieved using herbicides since the 1940s (Busi et al. 2013). Herbicide families, known as group B (Lee et al. 2011), such as sulfonylureas (SU) triazolopyrimidines (TP), pyrimidinyl-thiobenzoates (PTB), sulfonlyaminocarbonyl-triazolinones (SCT) and imidazolinones (IMI) were reported to inhibit acetohydroxyacid synthase enzyme (AHAS), also known as acetolactate synthase (ALS) (Duggleby and Pang 2000; Lee et al. 2011; Yaqoob et al. 2016). The respective enzymes AHAS and ALS are used judiciously as they involve different anabolic or catabolic roles in different organisms and may not be used interchangeably. AHAS generally catalyses the first step reactions of synthesis of leucine (Leu), valine (Val) and isoleucine (Ile) whereas ALS involves pathways in which 2-acetolactate is synthesized in species such as *Klebsiella pneumoniae* (Duggleby et al. 2008). However, AHAS or ALS is controlled by a single nuclear gene and transported to the chloroplasts after expression. AHAS expression levels generally were dependent on developmental and growth stages of plants and modulated constitutively. Additionally, AHAS enzymes were reported to be act more in meristematic tissues compared to other tissues. The regulation of balance of intracellular amino acid supply relies on BCA synthesis, modulated by AHAS, and BCA intermediates play important role in many metabolic pathways (Duggleby and Pang 2000).

The BCA synthesis is modulated by a common pathway in which the end products: Leu, Ile, and Val, are suppressed through feed-back inhibition during plant growth and development (Stidham 1991). On the other hand, AHAS inhibiting herbicides also can suppress the enzyme (Pang et al. 2002) by blocking substrate access channels in the enzyme. During blockade of these channels, while a part of the herbicide is fixed in the entrance of channel, the rest of it was pushed into the channel. Thus, the herbicides block AHAS substrates to access active site and suppress the enzyme activity (Pang et al. 2003). As a result of this mechanism, the AHAS inhibiting herbicides cause amino acid starvation of Leu, Ile, and Val and leads to the death of plants (Yu and Powles 2014).

The inhibitor herbicides are reported be used intensively in weed control since 1980s because of their wide-weed control spectrum, low mammalian toxicity (Lee et al. 2011; Zhang et al. 2017a), low use rates, high margins of crop safety and soil residue activity (Lee et al. 2011). However, upon intensive usage of these herbicides, some weeds have evolved resistance against inhibitor herbicides (Yu et al. 2010) through mutations of the AHAS gene which resulted in reduction of enzyme sensitivity (Pandolfo et al. 2016) or through rapid detoxification metabolism, also known as metabolic resistance (Tranel and Wright 2002). In order for a mutation to be effective against AHAS inhibiting herbicides, the mutations should occur where inhibitor substrates (i.e., herbicides) contact the enzyme (Pang et al. 2003).

AHAS herbicide mutations were generally identified as one or more point mutations (Bernasconi et al. 1995), leading to single amino acid substitutions in different points of polypeptide chain, thereby conferring resistance to herbicides (Tranel and Wright 2002). Point mutations were classified as target-site resistance mutations (TSR) (Yu and Powles 2014). Apart from target-site resistance mechanisms, some weeds can also impede enzyme inhibitors, partly or completely, to access their target organs (non-target site resistance mechanism-NTSR). Thus, either herbicidal inhibitors never reach the organs, or the transported inhibitors were below the lethal amount, i. e. not phytotoxic (Li et al. 2013). In this respect Ala122, Pro197, Trp574, Ser653 (Lee et al. 2011; Yaqoob et al. 2016), Ala205, Asp376, Arg377, Ser654 and Ser 627 (numbering refers to *Arabidopsis*) (Jimenez et al. 2016) were reported as most common amino acid substitutions endowing resistance against AHAS-inhibiting herbicides (Lee et al. 2011; Yaqoob et al. 2016; Jimenez et al. 2016). Furthermore, amino acid substitutions and their positions were reported to alter the level of weed resistance against commercial AHAS inhibitors and their types. Consequently modified AHAS enzyme(s), as a result of substitutions, were grouped as SU and TP resistant, IMI and PTB resistant, and SU, IMI, TP, and PTB resistant (Tranel and Wright 2002). The mutations in the AHAS enzyme also contributed to develop new herbicide resistant crops using different molecular and in vitro methods (Piao et al. 2017). Therefore, in this study, we aimed at providing insights into amino acid substitutions in the AHAS enzyme, causing resistance to AHAS inhibitor herbicides in the tomato (*Solanum lycopersicum)* and potato (*Solanum tuberosum*).

2. Material and methods

2.1. Retrieved AHAS sequence and analyses

The reviewed AHAS genes in *Arabidopsis* (AT3G48560.1) and rice (LOC_Os02g30630.2) were retrieved from the UniProtKB database (uniprot.org/) and both genes were blasted against protein sequences in *S. tuberosum* and *S. lycopersicum* in Phytozome v12.1.4 (phytozome.jgi.doe.gov/pz/portal.html; Goodstein et al. 2012). The identification of protein domains of investigated species were examined in the Pfam 31.0 database (http://pfam.xfam.org/; Finn et al. 2016). The physio-chemical features of amino acids were obtained from the ProtParam server (http://web.expasy.org/protparam/; Gasteiger et al. 2005). Subcellular localization (SL) of the AHAS proteins was predicted using the CELLO server (http://cello.life.nctu.edu.tw/; Yu et al. 2006). Further, the MEME server was employed for search of conserved motifs in the AHAS proteins with six motifs and in a range of 6-50 motif width (meme-suite.org/tools/meme; Bailey et al. 2009).

2.2. Phylogenetic and conserved motif analysis

AHAS enzymes of *Brassica rapa* (turnip), *Gossypium raimondii* (diploid cotton), *Medicago truncatula* (barrel clover), *Glycine max* (soybean), *Trifolium pretense* (red clover), *Brachypodium distachyon* (purple false brome), *Zea mays* (maize), *Setaria italica* (foxtail millet), *Panicum hallii* (panicgrass) and *Sorghum bicolor* (silage sorghum) were added to the phylogenetic tree with AHAS genes in *Arabidopsis*, rice, tomato, and potato to distinguish if there was a separation among dicot and monocot plants. A total of 14 AHAS sequences were aligned using the Bioedit V7.0.5 with the Clustal W method (Hall 1999). The tree and its distance matrix were constructed using the maximum likelihood (ML) method with 1000 bootstrap replicates (Jones et al. 1992) in MEGA 7 (Kumar et al. 2016).

2.3. 3D modelling of AHAS proteins

The Phyre^2 server was used at intensive mode for prediction of 3D proteins structures (sbg.bio.ic.ac.uk/phyre2/; Kelley et al. 2015). The predicted 3D structure of AtAHAS, OsAHAS, SlAHAS and StAHAS were validated using the Vadar server

(http://vadar.wishartlab.com; Willard et al. 2003). CLICK server (http://cospi.iiserpune.ac.in/click/; Nguyen et al. 2011) was employed for pairwise superimposition of AtAHAS, OsAHAS, SlAHAS and StAHAS protein structures. The comparison of investigated species was done based on overlap values.

2.4. Mutagenesis analyses

The StAHAS and SlAHAS enzymes were subjected to mutagenesis and docking analyses. Pdp files of investigated species were uploaded to the HotSpot Wizard (https://loschmidt.chemi.muni.cz/hotspotwizard/; Bendl et al. 2016) to find, point mutations, amino acids and their positions, which changed the enzyme properties. The degenerate codons were selected among those whose side chains directed towards either pockets or tunnels and the codon library was constructed using these codons with 10% minimal frequency from S. tuberosum and S. lycopersicum codon tables. Later, the prediction of protein stability of the enzymes in case of target site mutations was made by the CUPSAT server (http://cupsat.tu-bs.de; Parthiban et al. 2006) using pdp files of both species.

2.5. Docking procedure

Two ligands, Chlorosulfuron (CS) and Imazaquin (IQ) to represent sulfonylurea herbicides and Imidazolinone, respectively, were used in the docking procedure. Before docking, the ligands were optimized using AM1 base, set up in GAMESS-US software (Schmidt et al. 1993). Later, hydrogen (H) atoms were added to the proteins for docking analysis and StAHAS and SlAHAS proteins were docked using AutoDock 4.0 with the Lamarican genetic algorithm (Morris et al. 2009). The results were visualized on MGL Tools 1.5.6 software (Sanner 1999).

3. Results and discussion

3.1. Sequence analyses of AHAS genes and proteins

Relying on genome wide analysis in *Arabidopsis*, the identified *AHAS* genes in *Arabidopsis* (AtAHAS), rice (OsAHAS), tomato (SlAHAS) and potato (StAHAS) were presented in Table 1. All proteins were composed of three multiple domains: N-terminal TPP binding domain (PF02776), central domain (PF00205) and the C-terminal TPP binding domain of thiamine pyrophosphate enzyme (PF02775). Isoelectric points (*pI*) of the proteins were slightly acidic and varied between 6.1-6.5. Putative AHAS proteins molecular weights ranged from 69.39 to 72.58 kDa. AHAS encoding genes of the investigated species were located on the third chromosomes except for OsAHAS. All AHAS genes had three exons with open reading frames ranging from 1935 bp to 2013 bp. The AHAS proteins were found to be localized putatively in either chloroplast or mitochondria.

3.2. Conserved motif analyses of AHAS enzymes

The AHAS gene in Arabidopsis was blasted on selected 10 plant species from monocots and dicots. The alignment analysis showed that the AHAS proteins had highly well conserved residues across the species differing in length. On account of additions and deletions in non-conserved regions of the enzyme in various species, both the length of the enzyme and the positions of the conserved residues in the enzyme were reported to have been altered (Tranel and Wright 2002). To find the most conserved six motifs, a motif analysis was conducted using the MEME tool. As a result, five out of six motifs were found related to thiamine pyrophosphate (TPP). Motif 3 (ITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVMDVEDI PRVIREAFFL), motif 4 (DLLLAFGVRFDDRVTGKLEAFASRAKIVHIDIDSAEIGK NKQPHVSICAD), and motif 5 (PRKGADILVEALEREGVTDVFAYPGGASMEIHQALTRS NIIRNVLPRHEQ) were identified as N-terminal thiamine pyrophosphate (TPP) binding domain while motif 1 (DGSFIMNVQELATIKVENLPVKIMLLNNQHLGMVVQW EDRFYKANRAHTY) was the C-terminal TPP binding domain. Further, motif 6 (CLQSSDELRRFVELTGIPVASTLMGLGAFPTGDELSLQM LGMHGTVYANY), was determined as the central domain. Motif 2 (FKTFGEAIPPQYAIQVLDELTNGNAIISTGVGQHQMWAA QYYKYKKPRQW) was not associated with the domain structure in the Pfam database (Fig. 1). TPP was stated to be most important factor of all three co-factors of catalytic subunit of AHAS in which C2 atoms constitute the centre of active site and initiate catalysis (Duggleby et al. 2008). The presence of two highly conserved residues of the enzyme was reported as GDG and NN. These residues were found in motif 1 by not including the first G residue. GDG residues were suggested to be located at the N terminal end of α-helix and function in occurrence with the β-turn-αβ structural motif. Regarding the NN residues at the C terminal end, it was assumed to be bonded with the nitrogen atoms of thiamine in TPP (Hawkins et al. 1989). However, Trp, as a substrate recognition site of AHAS,

Table 1. The some selected features of AHAS proteins of *Arabidopsis*, rice, potato, and tomato.

Transcript ID (Phytozome)	Species	ORF (bp)	Chr no	Exon no	Protein Length (aa)	Domain family	Mol. wt. (kDa)	*pI*	SL
AT3G48560.1	*Arabidopsis*	2013	3	3	670	PF00205 PF02775 PF02776	72.58502	6.20	C
LOC_Os02g30630.2	*Oryza sativa*	1935	2	3	644	PF00205 PF02775 PF02776	69.39271	6.48	M
PGSC0003DMT400084507	*Solanum tuberosum*	1980	3	3	659	PF00205 PF02775 PF02776	71.97261	6.30	M
Solyc03g044330.1.1	*Solanum lycopersicum*	1980	3	3	659	PF00205 PF02775 PF02776	71.91252	6.16	M and C

ORF: Open Reading Frame, *pI*: Isoelectric point, SL: Subcellular Localization, C: Chloroplast, M: Mitochondria.

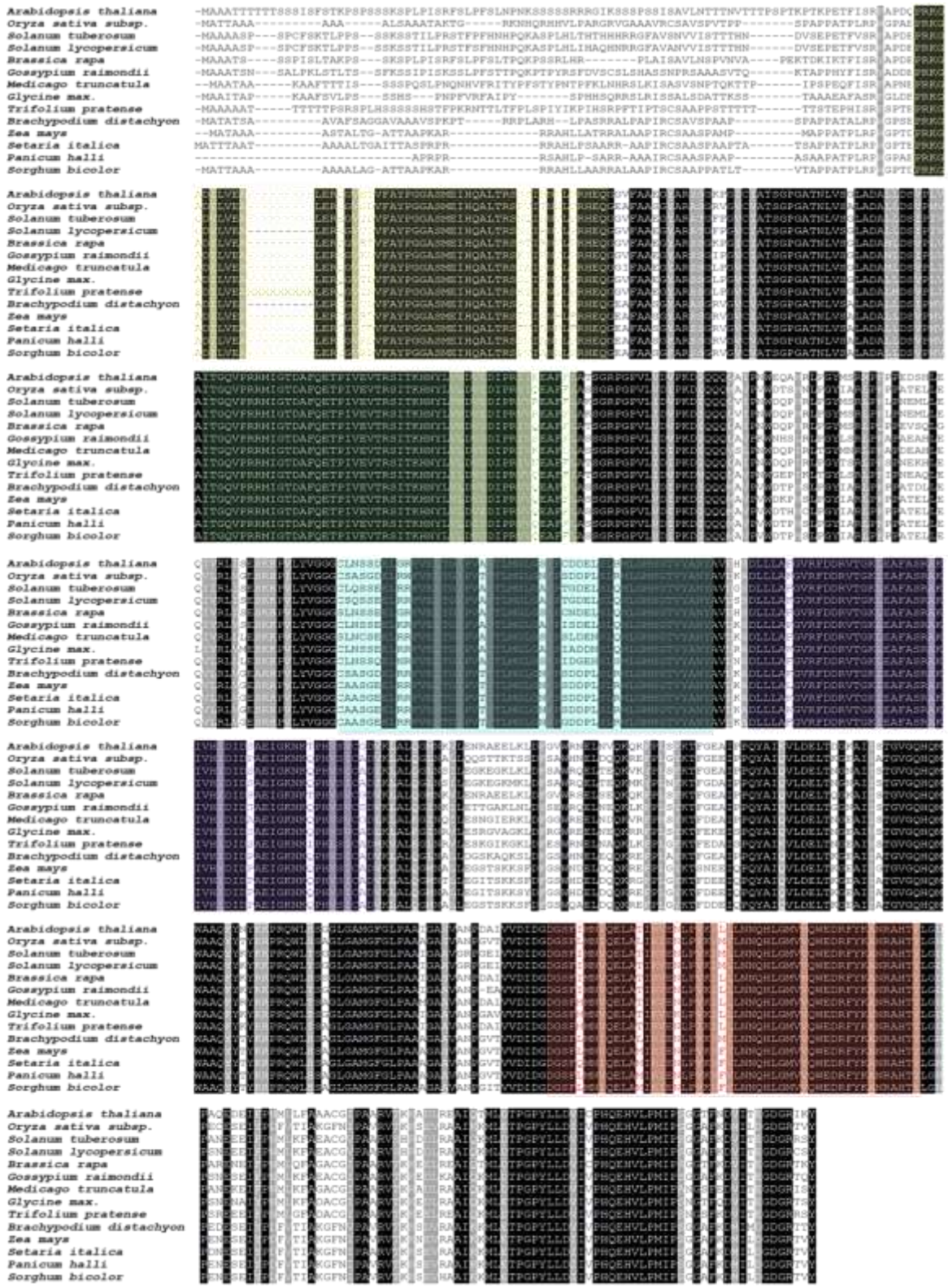

Figure 1. Conserved motifs of AHAS proteins of *Arabidopsis*, rice, tomato and potato. The yellow, light see green, green, purple and red stand for motif 5, motif 3, motif 6, motif 4, and motif 1 respectively. Since motif 2 was not associated with domain structure in the Pfam database, it was not shown. Similar and identical amino acids were shown in grey and black colours, respectively. Trp was found in motif 1, shown inside red rectangular area.

was found in motif 1 as conserved residue in all AHAS proteins (Fig. 1). In parallel to this, the residues (GDGSFIMNVQELATIRVENLPVKVLLLNN), to a greater extent and similar to motif 1, were defined as a co-factor or TPP binding domain. Moreover, His and Glu residues at the end of motif 5 were identified as catalytic glutamate (Duggleby and Pang 2000). The binding of glutamate to TPP via hydrogen bonds when TPP binds to AHAS, was suggested to be one of the important conserved features of AHAS (Duggleby et al. 2008). The AHAS proteins of cocklebur and corn mutants were also reported to have highly conserved residues to a large extent, similar to all motifs found in this study (shown as black and grey shading in Fig. 1) (Bernasconi et al. 1995). All these findings considered, the identical amino acids in conserved regions were highly involved in herbicide resistance, catalysis, substrate specificity, FAD and Mg^{+2} bindings.

3.3. *Phylogenetic analyses*

The AHAS protein sequences were used to construct a phylogenetic tree (Fig. 2). The results showed that the AHAS proteins in different species divided into two main groups as group A and group B, which are consisted of three subgroups (A1, A2 and A3) and two subgroups (B1 and B2) respectively. Dicot species were separated from monocots with 100 % bootstrap values and an orthologous homology was observed between them. The highest bootstrap values were found between SlAHAS and StAHAS, AtAHAS and Brassica at 100%. The members of Leguminosae and Gramineae families were clustered under A2 (92 %) and B2 (88 %) subgroups respectively. These findings agreed with those of Shimizu et al. (2011), suggesting that monocot and dicot separation was clearly observed among species.

3.4. *3D modelling of AtAHAS, OsAHAS, SlAHAS, and StAHAS*

The Phyre[2] server was employed for protein homology and analogy of, SlAHAS, and StAHAS. AtAHAS, whose crystal structure is known, was also included in the analysis to make a better comparison with OsHAS, SlAHAS and StAHAS. The validation of models was made through Ramachandran plot analysis and the secondary structure of models was calculated using the VADAR server. The secondary structure of AHAS proteins contained 34-37% α-helices, 20-22% β-strands, 40-45% coils, and 16-19% turns. Furthermore, based on a Ramachandran plot analysis, 97-98% amino acids of all the proteins were situated in core/allowed regions indicating that the models were good. These results were similar to those of Yaqoob et al. (2016), who stated that α-helices and β-strands are cover considerable part of AHAS proteins of *Arabidopsis* and rice with a 93% model probability validation for rice. To analyze model similarities and dissimilarities 3D models were superimposed on each other as pairs. The highest overlap values were obtained from pairwise superimpositions of AT3G48560.1-PGSC0003DMT400084507, LOC_Os02g30630.2 -PGSC0003DMT400084507 and LOC_Os02g30630.2 -Solyc03g044330.1.1 by 91.81%, 90.68%, and 90.06% respectively. The least structurally similar protein models were obtained from AT3G48560.1-LOC_Os02g30630.2, PGSC0003DMT400084507-Solyc03g044330.1.1, and AT3G48560.1- Solyc03g044330.1.1 pairwise superimpositions by 89.91%, 88.62%, and 88.32% respectively (Fig. 3). According to these results, the greatest similarity was found between AtAHAS and StAHAS, followed by those of OsAHAS and StAHAS. In our conserved motif and phylogenetic analyses we found that a considerable portion of the AHAS protein sequences were alike and SlAHAS and StAHAS were clustered under the same subgroup. However, this pattern was not confirmed by our protein homology analysis. This may stem from aggregation states of AHAS proteins giving rise to specific properties to the enzymes (Singh et al. 1991).

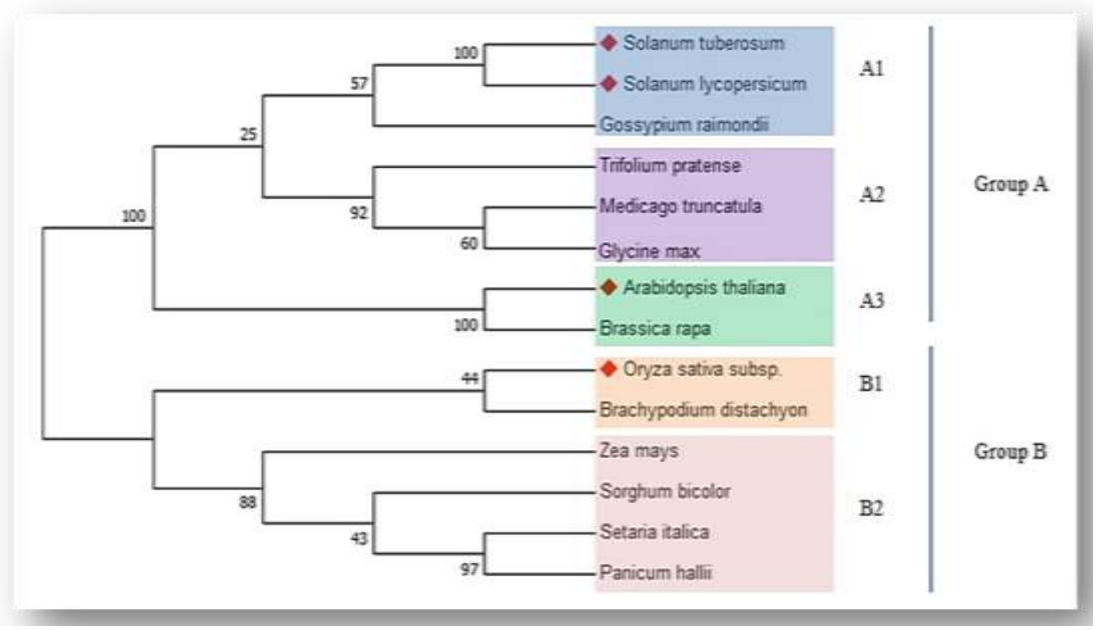

Figure 2. Phylogenetic tree of AHAS proteins of selected and investigated species. MEGA7 software was used for construction of the tree with maximum likelihood (ML) method with 1000 bootstrap replicates.

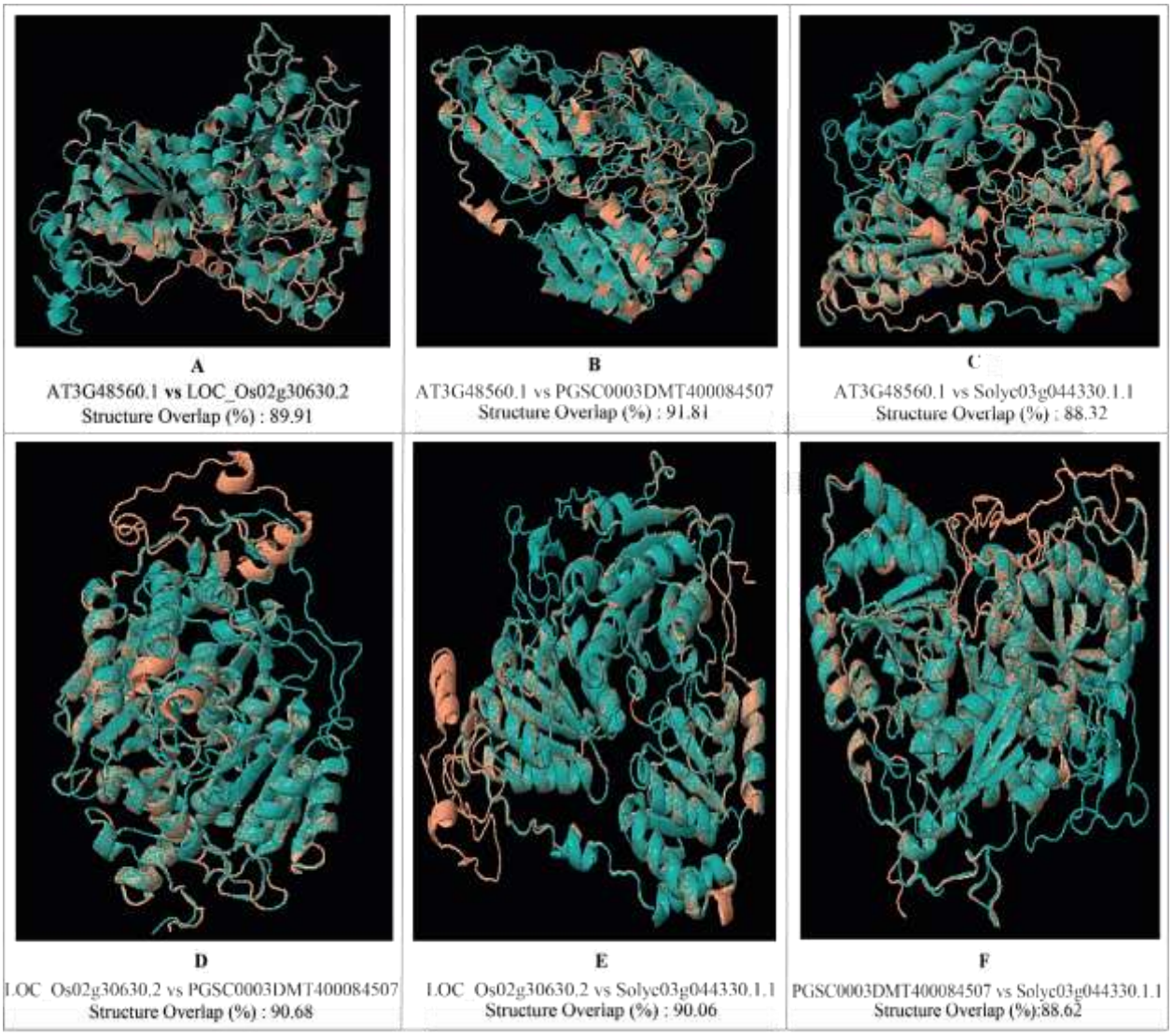

Figure 3. Pairwise superimposition of AHAS proteins of *Arabidopsis* (AT3G48560.1), rice (LOC_Os02g30630.2), potato (PGSC0003DMT400084507), and tomato (Solyc03g044330.1.1).

3.5. Mutation and docking analyses of StAHAS and SlAHAS

Some weeds naturally developed resistance to AHAS inhibiting herbicides through gene mutations (Piao et al. 2017). Fifty-four different species were reported to have resistance against AHAS inhibiting herbicides in various degrees through amino acid substitution at different codon positions (Menegat et al. 2016). The AHAS mutants can be developed by using different techniques such as chemical or site directed mutagenesis and clustered regularly interspaced short palindromic repeats (CRISPR) (Piao et al. 2017). To find suitable substitutions for any enzyme requires tiresome and costly screening processes of large sequences of protein libraries, supported by directed evolution experiments (Bendl et al. 2016). To address these concerns, problem mutation analyses of SlAHAS and StAHAS were conducted by means of computational tools. First of all, the positions of catalytically important amino acids in AHAS sequences, called hot spots were predicted and degenerate codons for these amino acids were identified using the HotSpot Wizard server. For this process, SlAHAS and StAHAS pdp files were uploaded to the HotSpot Wizard for the calculations and 48 pockets with 6 tunnels for StAHAS, and 51 pockets and 3 tunnels for SlAHAS were identified (see Fig. 4).

In addition to this, nine hot spots were detected in both SlAHAS and StAHAS. However, Lys592, Ile531, Gly578, Asp579, Glu595, and Gly598 residues in SlAHAS were excluded from the analysis as Asn123, Gln258 and Asn274 residues were not included in StAHAS since the side chains of these hot spots were not oriented towards tunnels or pockets. After identification of suitable and non-suitable degenerate codons for the detected hot spots, the only suitable residues were further analyzed via the CUPSAT server to ensure if they provide protein stability in case of substitution. As a result, Lys and Arg in StAHAS were identified as protein stabilizing residues for Ile124, while Leu was detected for Met266 and Ala; with Pro and Ser distinguished for Leu272 (Table 2). Similarly, Ala, Phe, Arg, and Val were mutable residues for Lys541, whereas Ile was distinguished for Val542 with regards to preservation of enzyme activity.

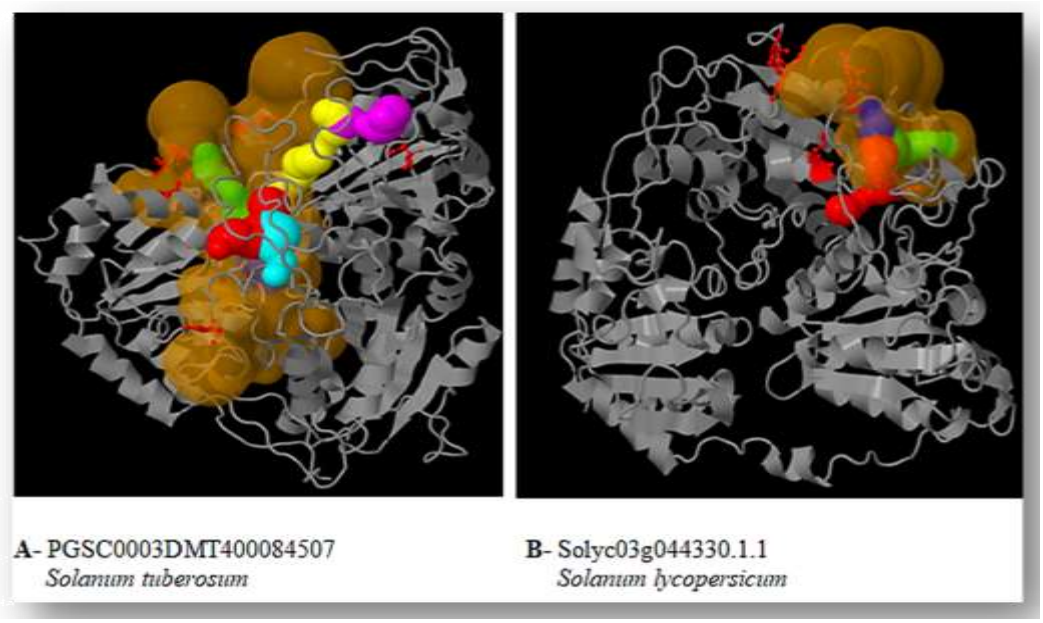

Figure 4. The predicted results of HotSpot Wizard for *S. tuberosum* (StAHAS) (panel A) and *S. lycopersicum* (SlAHAS) (panel B). One pocket and 6 tunnels for *S. tuberosum* and one pocket and three tunnels for *S. lycopersicum* were displayed in the panel A and B respectively. The same colour palette was used for the coloration of pockets and tunnels. Pocket one was shown in orange and the first, second and third tunnels were in purple, green, and red respectively on both panels. The fourth, fifth and sixth tunnels were displayed in cyan, yellow, and magenta respectively in panel A. The selected hot spot residues were shown with red balls and sticks in both panels.

Table 2. Mutable positions and residues for stability of AHAS proteins in investigated species.

Species	Hot Spots[a]	Mutability Grade[a]	Stabilizing Suitable Degenerate Codons[b]	Destabilizing Suitable Degenerate Codons[b]
S. tuberosum (StAHAS)	Ile124	8 (high)	Lys, Arg	Ser and Thr
	Met266	9 (high)	Leu	Ile, Lys, Asn, Arg and Val
	Leu272	9 (high)	Ala, Pro, Ser	Glu, and Lys
S. lycopersicum (SlAHAS)	Lys541	7 (high)	Ala, Phe, Arg, and Val	His and Lys
	Val542	7 (high)	Ile	Ala and Cys

[a] HotSpot Wizard Server was used for calculation of mutability score and identification of the most suitable degenerate codons. The residues numbers belong to StAHAS and SlAHAS and degenerate codons were searched in *S. lycopersicum* and *L. lycopersicum* respectively.
[b] Prediction of suitable degenerative codons were made on CUPSAT server.
Note: Numbering of residues in StAHAS and SlAHAS refer to the *S. tuberosum* and *S. lycopersicum* respectively.

As indicated before, the docking analyses were conducted using CS and IQ ligands for StAHAS and SlAHAS (Fig. 5). Of nine conformations, predicted by AutoDock4, the minimum binding affinities were found as -7.18 (Ser387) and -7.03 (Arg235) kcal mol^{-1} for SlAHAS and CS ligand (Fig. 5a). Negative and minimum binding affinity indicates stronger binding affinity (Spratt and Greenwood 2000). Consequently, CS can be suggested as a good ligand candidate for SlAHAS. The free binding energy of CS ligand to amino acids in SlAHAS, from minimum to maximum, can be listed as Ser387, Arg235, His341, Asp364, Gly234, Phe76, Ile404, Gly297, Gly497, and Gln478 (Fig. 5a). As for IQ ligand and SlAHAS, the minimum binding affinity conformations were obtained as -8.06 and -7.98 kcal mol^{-1} (Fig. 5b). The putative contact amino acids for IQ in SlAHAS were ordered, from the lowest to the highest binding affinity, as Phe11, Ala40, His341, Arg366, Gln478, Ser175, and Arg235 (Fig. 5b). Collectively, His341 and Gln478 seemed to be mutable residues for both CS and IQ in SlAHAS.

Similarly the lowest binding affinity for CS was found as -8.17 and -7.11 kcal mol^{-1} for StAHAS (Fig. 5c). In this respect Lys232, Asn123, Arg53, Ser41, Val61, Met502, Val560, Met502, and Arg366 in StAHAS were residues to bind to CS through H binding in terms of increased free binding energy (Fig. 5c). Furthermore, the minimum binding affinity of IQ ligand was observed as -6.67 and -6.47 kcal mol^{-1} for Lys405 and Lys489 in StAHAS (Fig. 5d). All residues binding to IQ through H bounds were listed, from minimum to maximum, as Lys405, Lys489, Arg268, Ser387, Asn589, Val61, Arg491, Asn467, Lys488, and Arg366 in terms of binding affinity. On the whole, Val61 and Arg366 of StAHAS were found to be mutable sites for both ligands. Interestingly, Arg366 was observed as a mutable site in SlAHAS for IQ as well.

Mutable residues in AHAS endowing herbicide resistance were reviewed by various studies (Tranel and Wright 2002; McCourt et al. 2005; Duggleby et al. 2008; Vila-Aiub et al. 2009; Yu and Powles 2014). The most studied amino acid mutations depending on inhibitor herbicides were Pro-197, Asp-376, Trp-574 (Yu et al. 2010; Pandolfo et al. 2016; Li et al. 2013; Zhang et al. 2017a). Additionally, Ser653 (Lee et al. 2011), Ala122 (Li et al. 2013), Ala205 and Ser264 (Brosnan et al. 2016) mutations were investigated in various plants. Specifically, in weeds Zhang et al. (2017a) reported that Pro197Leu or Pro197His or Asp376Glu or Trp574Leu mutations in *Descurainia sophia L.* gives resistance to tribenuron-methyl. Moreover, Pro197Arg mutation in AHAS of *Capsella bursa-pastoris (L.) Medik* (Shepherd's purse) resulted in resistance against tribenuron-methyl and

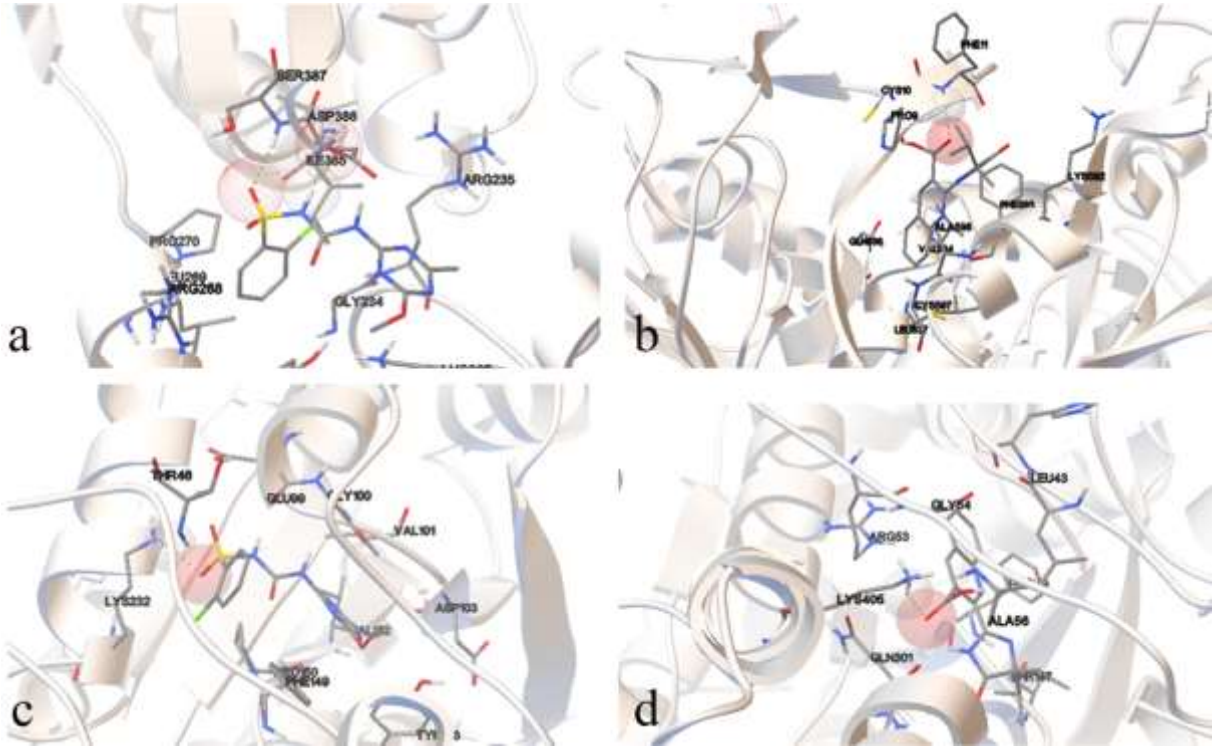

Figure 5. The docking results of AHAS enzymes with CS and IQ were shown in panel a, b, c and d. The result of docking CS ligand with SlAHAS. The H binding between Ser387 and CS was shown with red spheres (binding affinity was -7.18 kcal mol^{-1}) (a). The docking analysis of IQ ligand with SlAHAS. H binding between Phe11 and IQ was displayed with red region (the binding affinity was -8.06 kcal mol^{-1}) (b). The docking interaction of CS ligand with StAHAS. Lys232 was bind to CS ligand through H binding represented with red sphere (binding affinity was -8.17 kcal mol^{-1}) (c). Docking of StAHAS with IQ ligand. The red sphere shows H binding of Lys405 with IQ. (The binding affinity was -6.67 kcal mol^{-1}) (d).

flucarbazone-Na. This mutation also rendered Shepherd's purse moderately resistant to florasulam; low resistant to pyrithiobac sodium and pyroxsulam, and sensitive to imazethapyr (Zhang et al. 2017b). Similarly, Pro-197 mutation caused two biotops (R2 and R3) of wind bent grass (*Apera spica-venti L.)* to be resistant ALS herbicides and two other biotops (R4 and R6) to be resistant to both ALS and *acetyl-CoA carboxylase* (Accase) inhibiting molecules (Adamczewski and Matysiak 2012).

Our results have no similarity with the results of these respective studies. This can be explained below. Initially, IMIs and SUs have no structural similarity and have no similarity to substrates of the enzyme. Therefore, they act in independent sites of the AHAS. Secondly, SUs and IMIs have different inhibition capacities. Generally, SUs inhibition capacities are 100 times stronger than IMIs since these ligands interact with neighbouring residues by making many the same contacts (Duggleby et al. 2008). Therefore, it can be proposed that the effect of mutations differs depending on the mutation and its neighboring amino acids.

Mutations in the AHAS gene may lead to alteration of feed-back regulation, emerging or disappearing or evolving cross resistance, change in the enzyme's catalytic activity, fitness cost, and pleiotropic effects (Vila-Aiub et al. 2009). The mutable residues, endowing cross or specific resistance in AHAS, were suggested to be non-conserved sites for all herbicides and show variation depending on sites where herbicides make contact with substrate access channels (Thompson and Tar'an 2014). Contrary to this, a more probable assumption was put forward concerning the sites by stating that residues in the AHAS genes across the species were highly conserved, since they may have a key role in enzyme activity. Otherwise, AHAS activities cannot be maintained in herbicide resistant plants (McCourt et al. 2006). In this respect, the enzyme inhibition was assumed to be associated with different but overlapping residues in which inhibitor herbicides act (Duggleby et al. 2008). In general, it can be suggested that mutable residues in these different but overlapping sites of the enzyme may influence the enzyme's properties, functions and activity.

4. Conclusion

In this research, we sought to develop further insights into putative mutable sites of AHAS enzymes in the tomato and potato. The predicted sites may give herbicide resistance to the investigated species. However, it should be understood that the mutation of any residues in AHAS may carry a fitness cost, negative or positive pleiotropic effects, and specific or cross resistance to herbicides. The most important approach in mutation studies should be rested on natural mutagenesis residues observed in different species since such mutations are not lethal although they may have pleiotropic effects, or resistance costs. Also, sensitivity of the AHAS enzyme to herbicides is mostly specific. Namely, mutations endowing resistance to SUs may not bring out resistance for IMIs. Thus, herbicide resistance studies should be conducted according to types of herbicides. To conclude, the results of our study may contribute to studies which would improve herbicide resistance of tomato and potato plants.

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