

Amino acids conferring herbicide resistance in tobacco acetohydroxyacid synthase

Dung Tien Le,¹ Jung-Do Choi² and Lam-Son Phan Tran^{3,*}

¹Research Team for Vector-Borne Diseases; National Agriculture Research Center; Tsukuba, Ibaraki Japan; ²Department of Biochemistry; Chungbuk National University; Cheongju, Chungbuk Korea; ³RIKEN Plant Science Center; Signaling Pathway Research Unit; Tsurumi, Yokohama Japan

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Abbreviations: AHAS, acetohydroxyacid synthase; FAD, flavine adenine dinucleotide; GST, glutathione S-transferase; IM, imidazolinone; SU, sulfonylurea; ThDP, thiamin diphosphate; TP, triazolopyrimidine

Acetohydroxyacid synthase (AHAS) (EC 4.1.3.18) is a target of commercially available herbicides such as sulfonylurea, imidazolinone and triazolopyrimidine. In plants and microorganisms, AHAS catalyzes the first common reaction in the biosynthesis pathways leading to leucine, isoleucine and valine. Intensive studies using different approaches—including site-directed mutagenesis, molecular modeling and structural analysis—on plant AHAS-s have contributed to the understanding of the herbicide-AHAS interaction. Knowledge of the critical roles of amino acid residues of plant AHAS in conferring herbicide resistance will enable the creation of new herbicide-tolerant AHAS which could be used to develop herbicide-resistant transgenic plants. Moreover, such information will also elucidate design strategies for more efficient herbicides that could also kill weeds resistant to previously used AHAS-inhibiting herbicides. In this review, we summarize the results of intensive searches for amino acid residues and their substitutions that confer herbicide resistance in tobacco AHAS.

Introduction

Herbicides are extremely effective tools for weed management. They work by interfering with crucial biochemical pathways in plants. More than 270 herbicides currently available in the market act by inhibiting one of the 17 herbicidal targets. Acetohydroxyacid synthase (AHAS) together with Photosystem II and Protoporphynogen IX oxidase is the targets of more than 40% of commercial herbicides.¹ AHAS (also referred to as acetolactate synthase—ALS) is an enzyme catalyzing the first common step in the biosynthesis of branch chain amino acids in bacteria, yeast and higher plants.² It is the target of several classes of structurally unrelated herbicides including the sulfonylureas (SU),¹⁻³ the imidazolinones (IM),⁶ the triazolopyrimidines (TP),⁷ the pyrimidyl-oxy-benzoates,^{8,9} the pyrimidyl-thio-benzenes⁹ and

the 4,6-dimethoxypyrimidines.¹⁰ The herbicide-resistant crops usually carry a mutant version of AHAS which is insensitive to the herbicides. Studies on AHAS have attracted a great deal of effort from the research community in the last few decades.¹¹ The structural aspects of reaction mechanism were studied in detail and reviewed in references 12 and 13. The active unit of the enzyme is composed of a homodimer with two active-sites positioned on the dimer interface, in which catalytically important residues are contributed by both subunits.¹³ AHAS requires thiamin diphosphate (ThDP), flavine adenine dinucleotide (FAD) and a divalent ion as cofactors.¹¹ Recently, the structures of Arabidopsis AHAS bound with five different SU-s and an IM were also determined.¹⁴ The structural studies revealed that SU and IM inhibit the AHAS enzyme by blocking the “substrate channel.” In addition, it also revealed from the structures that there are several amino acid residues that interact with all classes of herbicides and there are other residues that interact with only one class.¹⁴ A detailed analysis on binding and inhibition of the herbicides from a structural perspective revealed 16 amino acid residues involved in the interactions with SU, among which four residues adopted different conformations depending on which SU is bound.¹⁵ More recently, crystal structures of plant AHAS in complexes with two new sulfonylurea herbicides were also determined, confirming that the new herbicides also inhibit the enzyme by blocking the substrate tunnel.¹⁶

An understanding of the residues playing a critical role in conferring herbicide resistance will not only help in creating a new version of herbicide-tolerant AHAS, which could be used to make herbicide-resistant transgenic plants, but also shed light on the design of more efficient herbicides that could kill weeds resistant to previously used AHAS-inhibiting herbicides.

In addition to the application in agriculture, studies on AHAS inhibition also provide potential benefits in controlling diseases caused by intracellular bacteria. For example, Grandoni et al. reported that plant AHAS inhibitors could reduce the ability of *Mycobacterium bovis* to survive in the lungs and spleen of mice.¹⁷ Choi et al. and Sohn et al. found that inhibitors of recombinant AHAS of *Mycobacterium tuberculosis* could inhibit the growth of the bacterium.^{18,19} A research group in India reported that inactivation of *ilvB1* gene—which encodes for AHAS—in *M. tuberculosis* could attenuate its virulence in mice.²⁰

*Correspondence to: Lam-Son Phan Tran; Email: tran@psc.riken.jp
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Table 1. Amino acids conferring herbicide resistance in tobacco aceto-hydroxyacid synthase

<i>N. tabacum</i> *	<i>A. thaliana</i> numbering*	<i>S. cerevisiae</i> numbering*	SU	IM	TP
A121T	A122	A117	+++	+++	-
H142K	H143	H138	++	++	-
S167R	S168	S163	+++	-	-
F205	F206	F201			
F205A			+++	-	-
F205H			+++	+++	+++
F205W			+++	+++	-
F205Y			+++	-	+++
K255	K256	K251			
K255F			+++	+++	+++
K255Q			+++	+++	+++
M350C	M351	M354	++	+++	+++
H351	H352	H355			
H351F			+	+	-
H351M			+++	+++	-
H351Q			++	++	+
D374	D375	D378			
D374A			++	++	-
D374E			+	-	-
D375	D376	D379			
D375A			+++	-	+

*Numbering differences exist among corresponding amino acids in tobacco, *A. thaliana* and *S. cerevisiae*. SU, sulfonyleurea; IM, imidazolinone; TP, triazolopyrimidine. (+++) strong tolerance (IC_{50} not detectable up to 1 μ M for Londax or N311 (SU), and 1 mM for Cadre (IM) and TP. (++) moderate tolerance (IC_{50} at least 10 times higher than that of wild type). (+) somewhat tolerant. (-) Sensitive or insignificant tolerance.

Analyses of the function of AHAS in plants to identify essential amino acids faced difficulties because the enzyme is present in low concentrations and is unstable for purification and functional study. To overcome this problem, Chang et al.²¹ cloned and expressed tobacco (*Nicotiana tabacum*) AHAS in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST) to facilitate affinity purification of the enzyme. This strategy enabled the screening for functionally important amino acid residues in the enzyme by site-directed mutagenesis.

Studies on AHAS have attracted a number of groups across the globe. Interests in AHAS have focused not only on understanding its catalytic mechanism but also on exploring the amino acid residues, which play key role in herbicide binding and herbicide resistance. In this review we summarize recent achievements in the studies of tobacco AHAS with respect to herbicide binding and inhibition, and compare the findings with those reported for AHAS-s from other species.

Selection of Amino Acid Residues for Mutagenesis

Figure 1 shows the multiple sequence alignment of AHAS sequences from tobacco, Arabidopsis and yeast (*Saccharomyces cerevisiae*).

Table 1. Amino acids conferring herbicide resistance in tobacco aceto-hydroxyacid synthase

D375E			+++	-	+++
S506R	S507	S519	+++	+++	+++
S539	S540	S552			
S539A			+++	+++	+++
S539F			-	+++	+++
S539R			+++	+++	+++
M569C	M570	M582	+++	++	+++
V570	V571	V583			
V570I			-	-	-
V570Q			+++	-	+++
W573F	W574	W586	+++	+++	+++
F577	F578	W590			
F577D			+++	+++	+++
F577E			+++	+	+++
F577I			-	-	-
F577K			+++	+++	-
F577R			+++	+++	-
F577W			-	-	-
F577Y			-	-	-
S652	S653	G657			
S652C			-	-	-
S652T			-	+++	-

*Numbering differences exist among corresponding amino acids in tobacco, *A. thaliana* and *S. cerevisiae*. SU, sulfonyleurea; IM, imidazolinone; TP, triazolopyrimidine. (+++) strong tolerance (IC_{50} not detectable up to 1 μ M for Londax or N311 (SU), and 1 mM for Cadre (IM) and TP. (++) moderate tolerance (IC_{50} at least 10 times higher than that of wild type). (+) somewhat tolerant. (-) Sensitive or insignificant tolerance.

Selection of the amino acid residues in tobacco AHAS for mutational studies was performed based on the following criteria: selected amino acids should (1) have functional group at the side-chain; (2) be conserved across several AHAS sequences; and/or (3) be homologous to residues reported to have function in AHAS of other species. Table 1 summarizes those amino acid residues in tobacco AHAS, whose mutations have been shown to confer herbicide resistance, with cross-reference to corresponding amino acids in Arabidopsis and yeast AHAS-s.

Arginine residues. Having a positively charged group at the sidechain, arginine (Arg) plays important roles in proteins and enzymes. There are five identical Arg residues in AHAS sequences of tobacco, Arabidopsis and yeast. Three Arg residues of tobacco AHAS (R141, R372 and R376)—among which one is moderately conserved (R141) and the other two are highly conserved among plant AHAS-s—were selected for mutational study.²² Seven mutated AHAS-s were created: R141A, R141F, R141K, R372F, R372K, R376F and R376K. All the mutants appeared to be altered greatly in terms of catalytic functions. However, the in vitro inhibition by three classes of herbicides (Londax—a sulfonyleurea, Cadre—an imidazolinone and TP—a triazolopyrimidine) was impaired insignificantly. In structural

analysis, Duggleby et al. found that R376 (R377 in Arabidopsis numbering) provided key interaction to SU.¹⁵ In our study, substitution of R376 by Lys did not alter the enzyme's sensitivity to herbicides. Our result suggested that Lys could provide similar herbicide interaction as that provided by the Arg residue.

Aspartic acid residues. An analysis of 17 AHAS sequences from plants and algae showed that the average number of aspartic acid residues was approximately 33. Among these, 9 residues are identical in 17 sequences. Multiple sequence analysis revealed that two aspartic acid residues are located consecutively in the β -domain of tobacco AHAS in a highly conserved motif (₃₇₂RFDDR₃₇₆). In their study, Le et al.²³ constructed four single-point mutants (D374A, D374E, D375A and D375E) and two double mutants (D374A/D375A and D374E/D375E) at these two residues of tobacco AHAS. The authors found that the mutated tobacco AHAS enzymes were strongly resistant to herbicides SU and TP, but insignificantly resistant to IM (Table 1). Mutations of the corresponding residues in yeast AHAS were also reported to confer herbicide resistance.²⁴ Interestingly, although the four single-point mutations made the mutated tobacco AHAS-s strongly tolerant of herbicides, they did not alter the kinetic properties of the mutated AHAS enzymes.²³

Cysteine residues. In a protein, cysteine residues may be involved in stabilizing structure via disulfide bridges, or may form covalent bonds to ligands. Four cysteine residues (C163, C309, C411 and C607) were selected for our study with tobacco AHAS as they were found to be identical in both tobacco and Arabidopsis AHAS-s (Fig. 1). Nonetheless, their locations were not conserved in yeast AHAS sequence. Among five AHAS mutants created, only two were active (C411S and C607S). However, these mutants did not exhibit resistance to the tested herbicides. Thus, residues C411 and C607 are not involved in interaction with herbicides.²⁵ In another study, Chong et al. confirmed that although mutations of C163 and C309 residues did not confer herbicide resistance, these residues were indeed implicated in the formation of a disulfide bond.²⁶

Histidine residues. Three histidine residues (H351, H392 and H487) were found to be conserved in plant AHAS sequences, and thus, were selected for experimental studies of tobacco AHAS.²⁷ Six AHAS mutants (H351M, H351Q, H351F, H392M, H487F and H487L) were created and successfully expressed as soluble forms. The H487F mutation totally abolished the enzymatic activity under various assay conditions. We also replaced the His487 residue with Leu, which has a hydrophobic R group. The H487L mutant did not show enzymatic activity either under various assay conditions. The H392M mutant showed similar K_{app}^1 values for the three herbicides to those of wild type AHAS. Each mutation—whether H351M or H351Q or H351F—exhibited a large effect on the sensitivity to the three herbicides. The H351M mutant is highly resistant to two classes of herbicides, Londax and Cadre, but not to TP. In addition, the H351Q mutant was also considerably tolerant of Londax and Cadre, but showed limited resistance to TP. The mutation of H351F increased resistance somewhat to Londax and Cadre, but not to TP.²⁷ In another independent study, we

also found that the mutation of H142K could result in moderate resistance to SU and IM but no resistance to TP (Table 1).²⁸

Lysine residues. Multiple sequence alignment of tobacco, Arabidopsis and yeast AHAS-s revealed that residues K219, K255 and K299 in tobacco AHAS were well conserved (Fig. 1). Yoon et al. created four mutants (K219Q, K255F, K255Q and K299Q) to investigate their functions.²⁹ The mutated AHAS carrying K219Q substitution was found to be inactive, and the K299Q substitution exhibited similar sensitivity to Londax, Cadre and TP as that of the wild type AHAS. Both substitutions at residue K255 (K255F and K255Q) resulted in strong tolerance to the three tested herbicides (Table 1). In a structural study of Arabidopsis AHAS, residue corresponding to tobacco K255 (K256 in Arabidopsis) was found to contribute critical interaction to SU and its sidechain adopted different conformations depending on which sulfonylurea was bound.¹⁵

Methionine residues. Duggleby and Pang¹¹ previously reported that there are six highly conserved methionine residues in AHAS sequences which were identified in several organisms, from bacteria to yeast and plants. Study on AHAS from *A. thaliana* suggested that residues M124 and M351 are located at or near the herbicide binding site.¹¹ In our work with tobacco AHAS, we selected three well-conserved methionine residues M350, M512 and M569 for mutagenesis.³⁰ These residues were individually substituted by valine and cysteine. For M569, additional substitution by alanine was also included. We found that all mutations substituted by either valine or alanine inactivated the enzyme. Meanwhile, mutants substituted individually by a cysteine were active, although their kinetic properties were severely altered. Of these active mutants, M512C mutant did not exhibit tolerance to any of the tested herbicides; in fact, this mutant becomes more sensitive to TP. M350C and M569C mutants showed strong tolerance to Londax, Cadre and TP (Table 1). These data suggest that the herbicide binding site and active site in AHAS are partially overlapped.

Tryptophan residues. Three Trp residues were found to be identical (W439, W490 and W573) in AHAS sequences of tobacco, Arabidopsis and yeast. In addition, two other Trp residues (W266 and W503) were also identified as conserved amino acids in the plant AHAS-s but not in that of yeast (Fig. 1). Furthermore, the corresponding residue of W573 in Arabidopsis was previously reported to be involved in herbicide resistance.¹¹ These five residues (W266, W439, W490, W503, W573) of tobacco AHAS were selected for substitution to phenylalanine. Except the W490F mutant, all other mutants were active. However, only the mutant carrying W573F substitution was strongly resistant to Londax, Cadre and TP (Table 1), while the other active mutants (W266F, W439F and W503F) did not show a significant difference in herbicide sensitivity compared to the wild type enzyme.³¹ Our observations are consistent with the results of a structural study reported recently by Duggleby et al. which demonstrated that residue W574 in Arabidopsis provides the single most crucial interaction with SU, in which the indole ring stacks onto the heterocyclic ring at a distance of 3.5 Å.¹⁵

Other residues conferring herbicide resistance. In the search for herbicide-resistant AHAS mutants, Jung et al. selected three

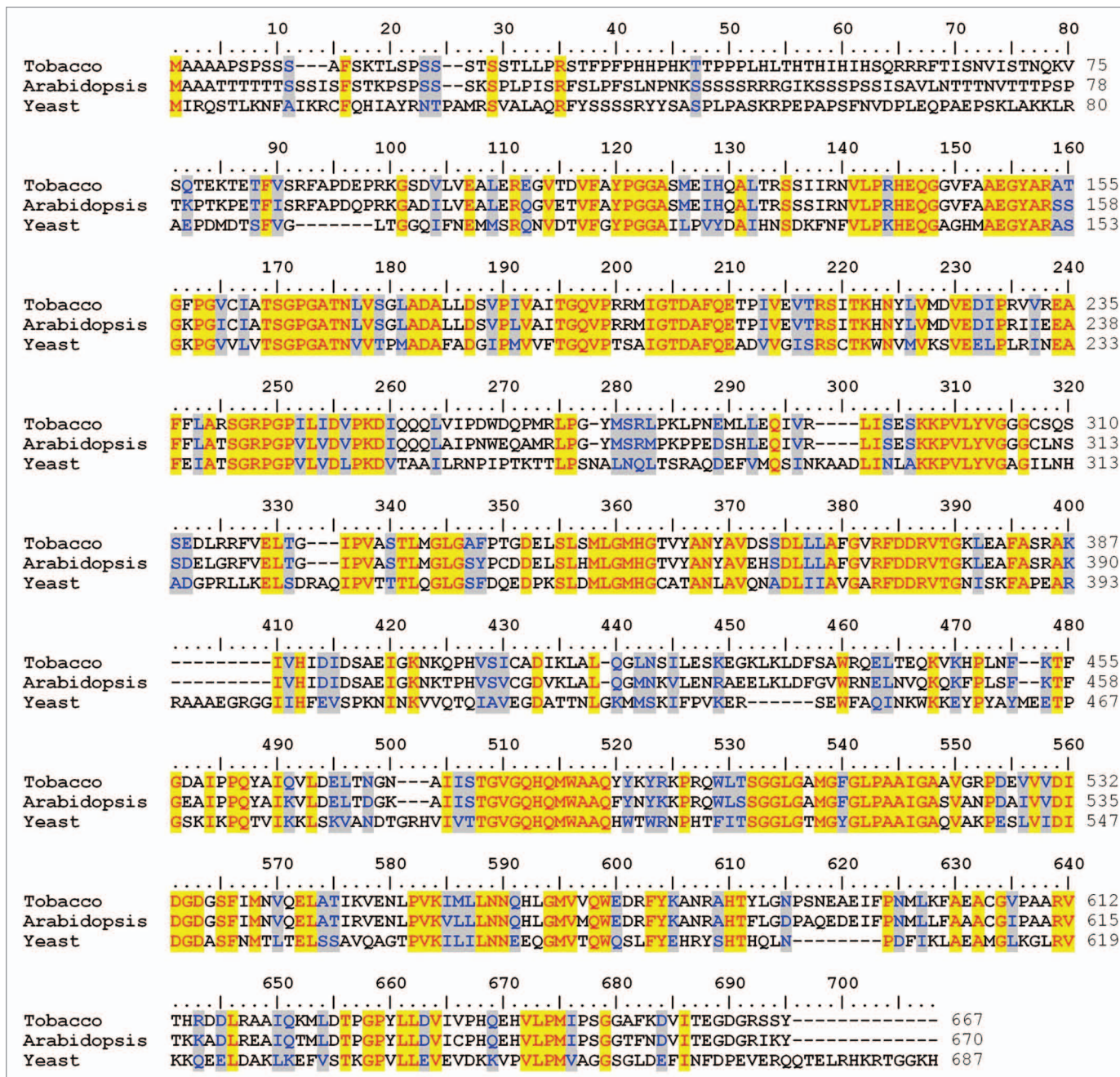


Figure 1. Multiple alignment of AHAS sequences from tobacco (SuRA), *Arabidopsis thaliana* and yeast. Identical residues are in red, similar residues are in blue.

residues of tobacco AHAS (F205, V570 and F577) for mutagenesis based on multiple alignment of 39 AHAS sequences which revealed that besides residue F205, A204 is also identical in all the 39 AHAS-s.³² In *Arabidopsis*, residue A204 was reported to be involved in herbicide tolerance.¹¹ Residue V570 together with residue M569 were found to be identical in 38 AHAS sequences,³² and, as discussed above, previously we discovered that residue M569 is involved in herbicide resistance.³⁰ The residue F577 is highly conserved in 39 AHAS sequences (identical in 28 out of 39 AHAS sequences) and its corresponding residue in *Arabidopsis* was also reported to be involved in herbicide resistance.¹¹

The authors created four single point substitutions at residue F205 (F205A, F205H, F205W and F205Y), three substitutions at residue V570 (V570I, V570N and V570Q) and seven substitutions at residue F577 (F577D, F577E, F577I, F577K, F577R, F577W and F577Y).³² Of these, the V570N mutant was shown to be inactive under various assay conditions. Four substitutions on residue F205 resulted in active mutant enzymes. Their herbicide-resistant profiles changed depending on the substituted amino acids. For example, F205A mutant was strongly resistant to Londax (SU) but was insignificantly resistant to Cadre (IM) and TP. The substitution to His at this residue conferred strong

tolerance of all three tested classes of herbicides. However, the substitution to either Trp or Tyr improved resistance to only two of the three classes of herbicides. F205W mutant was strongly tolerant of SU and IM but not TP; F205Y mutant was strongly resistant to SU and TP but not to IM. Of the two active mutants carrying substitutions at residue V570, V570I mutant did not show any resistance to three tested herbicides. Meanwhile, the V570Q mutant was strongly resistant to SU and TP but not to IM. Among seven substitutions at residue F577, three (F577I, F577W and F577Y) did not significantly alter herbicide sensitivity. Substitution to a negatively-charged residue Asp resulted in strong tolerance of all three classes of herbicides. However, substitution to Glu (which is also negatively charged) could confer strong tolerance only to SU and TP but not to IM. Substitution to positively charged residues such as Lys or Arg resulted in mutants which are strongly resistant to SU and IM but not to TP (Table 1).³²

In another study, Chong et al. investigated the role of residues A121 and S652 which were commonly found in weeds resistant to AHAS-inhibiting herbicides¹¹ and demonstrated that substitutions at these residues also provided resistance to herbicides (Table 1).³³ Specifically, the substitution A121T resulted in strong tolerance to SU and IM but not to TP. The substitution S652T exhibited tolerance to only IM but not to the other two classes. However, the substitution S652C did not alter herbicide sensitivity of the mutant. A double mutant combined A121T and S652T (A121T/S652T) was strongly tolerant to three classes of herbicides tested in their study.³³ Yoon and Choi's groups in Korea reported that the residue serine 539 (S539) of tobacco AHAS is also involved in binding with herbicides. Mutations of this residue into either Ala or Arg resulted in mutants tolerant of all three classes of herbicide.³⁴

Mutations Conferring Herbicide Tolerance in Natural Weed Biotypes

The excessive uses of AHAS-targeting herbicides have created several weed biotypes resistant to herbicides. However, mutations often occur in the AHAS coding genes of the plants. At least 102 weed species were identified to be resistant to AHAS-inhibiting herbicides (www.weedscience.org, accessed date Oct 2, 2009). So far, common mutations reported in the herbicide-resistant weeds were discovered at five particular residues: A122, P197, A205, W574 and S653 (numbering according to Arabidopsis AHAS sequence). Recently, two additional mutations were also found in the herbicide-resistant weeds, namely D376 and G654.³⁵ The substitutions identified in the resistant biotypes were Thr for A122 and Ala, Arg, His, Thr, Leu, Ile and Gln for P197, Val for A205, Leu for W574 and Asn and Thr for S653, respectively. The equivalents of these residues in tobacco AHAS also exhibited herbicide resistance when substituted. However, several other

single-point mutations summarized in this review were not found among naturally occurring herbicide-resistant weed biotypes.

Concluding Remarks

AHAS is an ideal herbicide target owing to the fact that: (1) it is specific only in plants and microorganisms, (2) it can bind to various chemical groups of structurally unrelated inhibitors, (3) it presents in low concentration in plants, and (4) inhibition of AHAS can interfere in essential metabolic pathways in plants. The first AHAS-inhibiting herbicide was commercialized in 1982. Since then, the intensive use of such herbicides has caused the emergence of so-called "super weeds" which are tolerant to AHAS-inhibiting herbicides. However, this should not be taken as a motivation to phase out the use of AHAS-inhibiting herbicides; their use brings about great economic gain. Thus, researchers are now interested in pursuing different approaches in engineering herbicide-insensitive AHAS enzyme and synthesizing more potent and selective inhibitors. For this purpose, the information provided in this review will be useful.

From the site-directed mutagenesis data (Table 1) together with recent structural studies on herbicide binding and inhibition mechanisms in plant AHAS-s, it is apparent that there are several amino acid residues which can directly interact with the herbicides. It is also clear that each class of herbicides (SU, IM and TP) requires a different set of residues for interaction. Some residues commonly bind to three classes, others are unique to only one or two classes. Results of the site-directed mutagenesis also indicated that, even at the same residue, substitutions to different amino acids may result in resistance to different classes of herbicides. This information will be useful for the "target-site directed" synthesis of efficient and selective inhibitors.

Because different substitutions (even at the same residue) can confer resistance to different classes of herbicides, site-directed mutagenesis could be employed to create various mutated AHAS enzymes resistant to selective herbicide(s). These mutated AHAS genes could then be used to generate selective herbicide-resistant transgenic crops. Rotation of such genetically modified crops and the selective herbicides could help reduce pressure on the evolution of AHAS-inhibitor-resistant weeds. In addition, the herbicide-resistant AHAS could also be used as a non-antibiotic selectable marker in transgenic crop development.

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