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Resistance mechanism of HRT1, a novel tomato mutant, to acetohydroxyacid synthase (AHAS)-inhibiting herbicides

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Research Article

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Abstract

Tomato (*Solanum lycopersicum* L.) is extremely sensitive to inhibitors of acetohydroxyacid synthase (AHAS; also known as acetolactate synthase [ALS]). Utilizing ethyl methanesulfonate mutagenesis of seeds of the commercial tomato line 'M82', we developed a tomato mutant, HRT1, that showed high resistance to imidazolinone herbicides (which act by inhibiting AHAS) in the greenhouse and under field conditions. The activity of AHAS extracted from HRT1 was significantly less affected by imidazolinone herbicides than that from the parental line M82. Following imazapic treatment, no differences were found in the content of free branched-chain amino acids in HRT1 tissues as compared to a dramatic decrease in M82 tissues. No differences were found in the susceptibility of AHAS to sulfonylurea herbicides. A single point transition mutation of C to T in the *AHAS1* gene located on chromosome 3 was detected. This mutation resulted in substitution of alanine by valine at amino acid position 194, corresponding to 205-Alal in *Arabidopsis*. Ligand–protein contact analysis showed that replacement of alanine by the larger hydrophobic valine residue results in increased repulsion, hindering herbicide binding. Segregation analysis indicated that the resistance to imidazolinones in line HRT1 is due to a single recessive gene.

Introduction

In this paper, we present the results of our study of the resistance mechanism of the tomato (*Solanum lycopersicum* L.) mutant line HRT1, previously obtained by ethyl methanesulfonate (EMS) mutagenesis of seeds of the commercial tomato line 'M82'. The mutant was found to be highly resistant to the imidazolinone herbicides imazamox, imazapic, and imazapyr; pyrithiobac-sodium (pyrimidinylthiobenzoic acid group); and propoxycarbazone sodium (sulfonylaminocarbonyl triazolinone group) (Han et al., 2012). HRT1 did not differ from M82 in its response to the sulfonylurea herbicides trifloxysulfuron, sulfosulfuron, and chlorsulfuron (Dor et al. 2016).

Wide use of acetohydroxyacid synthase (AHAS)-inhibiting herbicides has resulted in the appearance of AHAS herbicide–resistant weed populations (Gaines et al. 2020; Owen et al. 2012). Nevertheless, AHAS-inhibiting herbicides remain important in the continuously decreasing repertoire of herbicides, due to their broad spectrum of weed control, low toxicity to mammals, high selectivity, and high activity. which allows for low application rates (Owen et al. 2012), and in particular, their effectiveness against broomrapes (*Orobanche* and *Phelipanche* species) (Dor et al. 2016).

In addition, resistance to AHAS-inhibiting herbicides has been obtained by mutagenesis in many crop lines, such as corn (*Zea mays* L.) (Newhouse et al. 1991), *Arabidopsis thaliana* L. (Haughn and Somerville 1986), sugar beet (*Beta vulgaris* L.) (Hart et al. 1992; Wright and Penner 1998), canola (*Brassica napus* L.) (Guo et al., 2022; Swanson et al. 1989), soybean [*Glycine max* (L.) Merr.] (Sebastian et al. 1989; Ustun and Uzun 2023), tobacco (*Nicotiana tabacum* L.) (Chaleff and Ray 1984), cotton (*Gossypium hirsutum* L.) (Chen et al. 2023; Rajasekaran et al. 1996), rice (*Oryza sativa* L.) (Croughan 1998; Piao et al. 2018), wheat (*Triticum aestivum* L.) (Chen et al. 2021; Pozniak and Hucl 2004), barley (*Hordeum vulgare* L.) (Lee et al. 2011), and chickpea (*Cicer arietinum* L.) (Galili et al. 2021). Imidazolinone herbicides act by inhibiting AHAS (Duggleby and Pang 2000; Iwakami et al. 2012; Owen et al. 2012; Schloss 1995), which is a key enzyme in the biosynthetic pathway of the branched-chain amino acids leucine, isoleucine, and valine. The consequent deficiency in these amino acids results in plant death (Han et al., 2012; Iwakami et al. 2012; McCourt et al. 2005). In most cases, resistance is associated with mutations in the catalytic large-subunit *AHAS* gene family resulting in the substitution of a single highly conserved amino acid residue in the channel leading to the

herbicide-binding site of the AHAS protein (Duggleby et al. 2008; Lonhienne et al. 2022b; Tranel and Wright 2002; Walsh et al. 2012). At least 18 amino acid residues have been identified in bacteria, fungi, or plants in which mutation provides resistance to AHAS-inhibiting herbicides. Among them, mutations to amino acid residues Ala-122, Met-124, Pro-197, Arg-199, Thr-203, Ala-205, Lys-256, Met-351, His-352, Asp-375, Met-570, Trp-574, Phe-578, and Ser-653 (numbering according to *A. thaliana*) have been reported to be involved in plants' resistance to imidazolinones (Duggleby et al. 2008; Galili et al. 2021). Mutations can lead to AHAS inhibitor cross-tolerance, and some lead to broad cross-resistance to all classes of AHAS inhibitors (Duggleby et al. 2008).

In this study, we identified a mutation in the *AHAS* gene in HRT1 tomato plants and characterized the sensitivity of the enzyme to the imidazolinone herbicides imazapic and imazapyr, as well as the sulfonylurea herbicides rimsulfuron and sulfosulfuron. The prevention of HRT1's death due to branched-chain amino acid starvation following imazapic treatment was also shown. To ascertain the heredity of the resistance trait in the HRT1 line and to determine whether these alleles are recessive or dominant, segregation analysis was conducted. Ligand–protein contact analysis further allowed us to explain the changes in binding forces after modification of the protein–ligand binding region.

Materials and Methods

Plant Material

Tomato (*Solanum lycopersicum* L.) seeds of 'M82' were obtained from Tarsis Agricultural Chemicals (Petah Tikva, Israel). HRT1, a tomato mutant that is highly resistant to imidazolinone herbicides, was obtained by EMS mutagenesis (Dor et al. 2016).

Determination of AHAS Activity

Response of the enzyme AHAS to the herbicides was determined in vivo using crude enzyme extracts isolated and partially purified from young M82 and HRT1 seedlings, as described in Dor et al. (2017). Stock solutions of the tested herbicides were prepared in tetrahydrofuran. Aliquots of these solutions were taken and dried in test tubes. Tetrahydrofuran without herbicides was used in control tubes. AHAS activity was expressed as percentage of the control treatment containing no herbicides. The experiment was conducted in four replicates. Final herbicide concentrations in the reaction mixture were: imazapic (Cadre®, 240 g ai L^{-1}, BASF, Research Triangle Park, NC, www.basf.com) and imazapyr (Arsenal®, 240 g ai L^{-1}, BASF) at 1, 5, 10, 50, 100, and 200 µM; sulfonylurea rimsulfuron (Titus®, 250 g kg^{-1}, Corteva Agriscience UK, Melbourn, Cambridgeshire, UK, www.corteva.co.uk) at 0.05, 0.1, 0.5, 1, 5, and 10 µM; and sulfosulfuron (Monitor®, 750 g ai L^{-1}, Monsanto, St Louis, MO, USA, www.monsanto.com) at 0.0001, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 µM.

Determination of Free Amino Acids

M82 and homozygote HRT1 plants were grown in 2-L pots in a greenhouse. At the 4-true-leaf stage, five plants of each line were sprayed with imazapic at a rate of 14.4 g ai ha^{-1}. After 3 wk, leaf samples of treated and non-treated plants were analyzed for free amino acids content. Extraction, derivatization, and amino acid analysis were conducted according to Dor et al. (2017). All tests were performed in five replicates.

Sequencing of AHAS Genes

The tomato genome contains three large *AHAS* genes located on chromosomes 3, 6, and 7 (Tomato Genome Consortium 2012). To determine the DNA sequences of the three *AHAS* genes, total genomic DNA was extracted from young leaves of M82 and HRT1 plants at the 4-true-leaf stage, as previously described in Fulton et al. (1995). All three *AHAS* genes of M82 and HRT1 plants were PCR amplified, and the amplified fragments were sequenced at Hylabs Laboratory (Rehovot, Israel, https://www.hylabs.co.il/). For mutation determination, the DNA sequences of the amplified fragments of each *AHAS* gene were compared to their corresponding wild-type (WT) sequences published by the Tomato Genome Consortium utilizing DNAMAN 4.2 (Lynnon Biosoft, San Ramon, CA, USA).

Segregation Analysis

To ascertain the heredity of the resistance trait in the HRT1 line and to determine whether these alleles are recessive or dominant, we grew 10 plants that were homozygous for the resistance mutation and 10 homozygous M82 plants. F_2 plants derived from a cross between these two lines followed by self-pollination were screened for resistance to imazapic in a greenhouse and under field conditions as follows: 75 plants (5 groups of 15 plants) were planted in 2-L pots in Newe Ya'ar soil (medium-heavy clay–loam soil containing, on a dry weight basis, 55% clay, 23% silt, 20% sand, 2% organic matter, pH 7.1), 1 plant per pot, in a greenhouse; an additional 74 plants were planted in an open field at Newe Ya'ar research center (32.70917°N, 35.17989°E). M82 and HRT1 plants (10 of each) were planted in the greenhouse and in the field (for a total of 40 plants) as positive a negative control. At the 6-true-leaf stage, five M82, five HRT1, and all F_2 plants were sprayed with imazapic at a rate of 24 g ai ha^{-1}. The other five M82 and five HRT1 plants were sprayed with water (control). The number of resistant and sensitive plants was evaluated visually 3 wk after treatment. Plant injury was assessed on a scale of 5 (healthy, no damage) to 1 (death). For the segregation pattern, Pearson's chi-square analysis with JMP5 software (SAS Institute, Cary, NC, USA) was used to evaluate the suitability of the single-gene model.

Ligand–Protein Contact Analysis

Ligand–protein contact analysis, which predicts the binding forces obtained after chemical modification of the protein–ligand binding region, was conducted using LPC software (Sobolev et al. 1999). The model for protein-structure prediction was built as described in Sobolev et al. (2005).

Statistical Analysis

Data on herbicide influence on AHAS activity were computed by nonlinear regressions using Sigma-Plot v. 11.01 (SPSS, Chicago, IL) as $Y = y_0 + \frac{a}{1+\left(\frac{x}{x0}\right)^b}$ for imazapic and sulfosulfuron, and $Y = \frac{a}{1+\left(\frac{x}{x0}\right)^b}$ for imazapyr and rimsulfuron. The amino acid content results were subjected to ANOVA using JMP Software v. 5.0 (SAS Institute). The data were separated by standard error of the mean (SEM) and compared by Student's *t*-test ($P < 0.05$).

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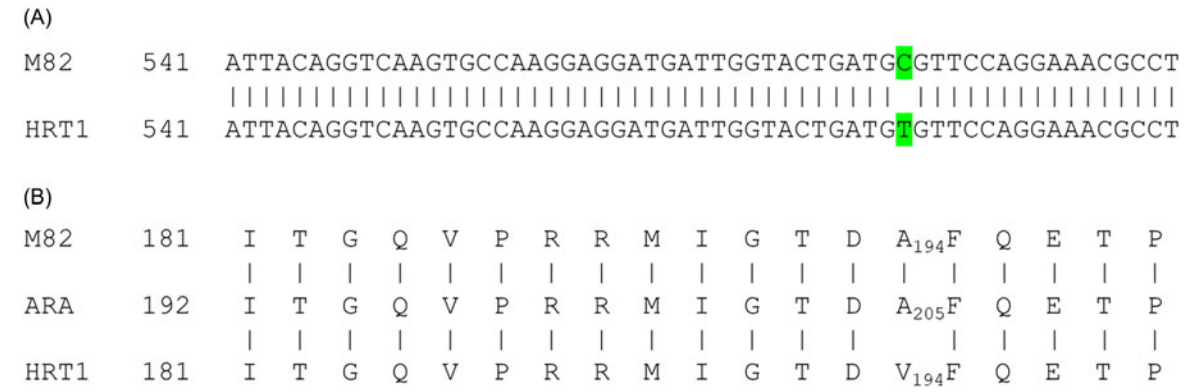

Figure 1. Sequence analysis of *AHAS1* located on chromosome 3. (A) *AHAS1* nucleotide sequences (541–597) of wild-type (WT) and HRT1 tomato. The C to T transition at position 581 is highlighted in green. (B) WT and HRT1 tomato AHAS1 amino acids 181–199 (192–210 according to *Arabidopsis thaliana* [ARA]). The alanine to valine transition at position 194 (205 according to *Arabidopsis*).

Results and Discussion

Determination of the Mutation

Mechanisms accounting for herbicide resistance in plants include increased metabolism, sequestration, reduced uptake and/or translocation, and modification of the herbicide target site (Lonhienne et al. 2022b; Sala et al. 2008). In most cases describing the resistance mechanism to AHAS-inhibiting herbicides, resistance is due to a point mutation(s) in the gene(s) encoding the AHAS catalytic subunit, reducing the enzyme's sensitivity to herbicides (Han et al. 2012; Lonhienne et al. 2022a, 2022b).

Sequence analysis of the three *AHAS* genes revealed a single point transition mutation of C to T in *AHAS1* at position 581 (Figure 1A) located on chromosome 3 (Tomato Genome Consortium 2012). This resulted in a substitution of alanine by valine at position 194, corresponding to Ala-205 in *Arabidopsis* (Figure 1B). This is a common mutation providing resistance to imidazolinones (Jain and Tar'an 2014), as reported for chickpea (Thompson and Tar'an 2014), sunflower (*Helianthus annuus* L.) (White et al. 2003), and the weeds redroot pigweed (*Amaranthus retroflexus* L.) and eastern black nightshade (*Solanum ptycanthum* Dunal) (Ashigh and Tardif 2007; Beckie and Tardif 2012; McNaughton et al. 2005). To the best of our knowledge, this is the first report of an alanine to Val-194 (according to *Arabidopsis* 205) mutation in imidazolinone-resistant tomatoes. No additional mutations were found in this gene, or in *AHAS2* or *AHAS3* located on chromosomes 7 and 6, respectively (data not shown).

Response of AHAS to Herbicides

The activity of AHAS extracted from HRT1 and M82 tomato lines in the presence of imidazolinone or sulfonylurea herbicides was determined in vitro using crude enzyme extracts. AHAS enzyme extracted from the parental tomato line M82 was considerably more sensitive to imazapic than that extracted from HRT1 (Figure 2A). The activity of the M82 enzyme was already significantly decreased at 1 µM imazapic, a concentration that did not affect the AHAS extracted from HRT1; complete inhibition of the M82 enzyme was obtained at 10 µM, whereas the HRT1 enzyme was still active at 100 µM; LD_{50} for M82 was 0.47 µM compared with 3.31 µM for HRT1. M82 AHAS was also more sensitive to imazapyr than the HRT1 enzyme (Figure 2B); LD_{50} for M82 was 2.52 µM compared with 6.65 µM for HRT1. On the other hand, AHAS enzymes of both lines were extremely sensitive to the sulfonylurea herbicides rimsulfuron and sulfosulfuron (Figure 2C and 2D). LD_{50} values were 0.02 and 0.04 µM for rimsulfuron and 0.0008 and 0.0018 µM for sulfosulfuron, for M82 and HRT1, respectively. The HRT1 resistance to the imidazolinone group herbicides was thought to be due to a change in the herbicide's target site on the AHAS protein. Interestingly, at all rates of imidazolinones, AHAS of HRT1 retained its activity at 25% to 45% of the control, indicating that only one of the three AHAS enzymes had become resistant. Resistance caused by point mutations in the *AHAS* gene may be specific to imidazolinone herbicides, to sulfonylurea herbicides, or to a broad spectrum of AHAS inhibitors (McCourt et al. 2006). For example, substitutions of Pro-197 usually provide resistance to sulfonylurea but not imidazolinones, whereas substitutions of Ala-122 result in imidazolinone but not sulfonylurea resistance. In many cases, alterations of Ala-205 to Val-205 have been reported to provide resistance to both groups of herbicides (Saari et al. 2018; Tranel and Wright 2002). Both the sulfonylureas and imidazolinones inhibit the enzyme by binding within and obstructing the channel leading to the active site. However, only 10 amino acid residues are involved in the binding of both sulfonylureas and imidazolinones. The other residues interact only with sulfonylureas or only with imidazolinones. Thus, the binding sites of the two classes of herbicides only partially overlap (McCourt et al. 2006). Unfortunately, we did not find any resistance of the HRT1 mutant to sulfonylurea, aside from partial resistance to foramsulfuron (Equip®, 22.5 g ai L^{-1}, Bayer AG, Leverkusen, Germany, www.bayer.com/) (Dor et al. 2016).

Influence of Imazapic on Contents of Total and Branched-Chain Amino Acids in Plant Tissues

Three weeks after being sprayed with imazapic, total amino acid content was significantly reduced in leaves of M82, from 2,984 nM g^{-1} to 1,964 nM g^{-1}, but not in leaves of HRT1 (Figure 3A). In a previous study, imazapic significantly reduced total free amino acids in *Phelipanche aegyptiaca* (Pers.) Pomel. plants attached to HRT1 plants, but not in the roots of the HRT1 plants (Dor et al. 2017). A reduction in total free amino acids was also obtained in the leaves and roots of imazethapyr-treated pea (*Pisum sativum* L.) (Zabalza et al. 2013), and in canola treated with ZJo273 (a novel AHAS inhibitor) (Tian et al. 2014). Similar observations were made for the total content of branched-chain amino acids (Figure 3B) and for isoleucine (Figure 3C) and valine (Figure 3D) content: following imazapic treatment, total branched-chain amino acids in leaves of M82 plants were significantly reduced from 344 to 258 nM g^{-1}; isoleucine content was significantly reduced from 102 to 68 nM g^{-1}, and valine content

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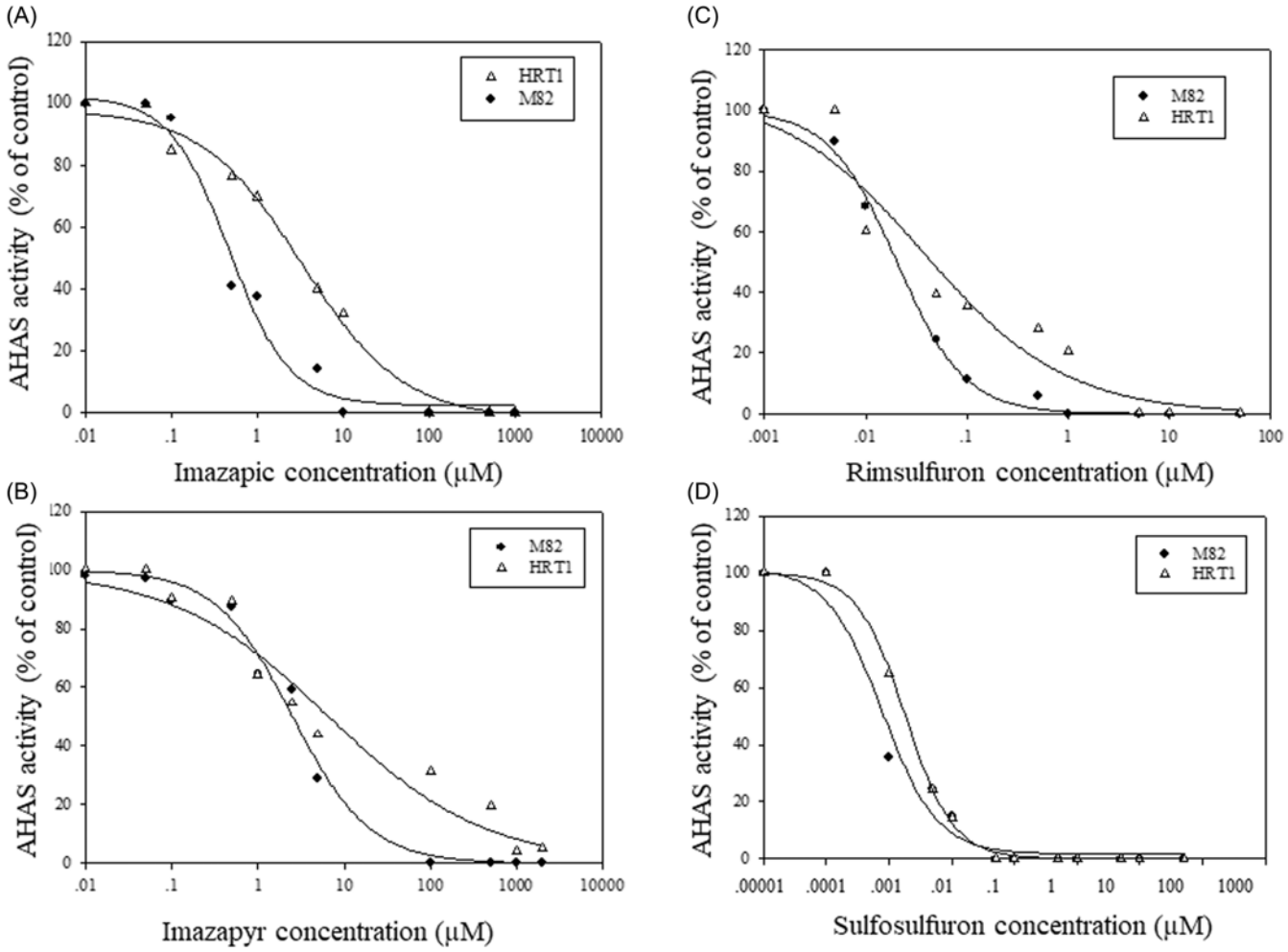

Figure 2. Influence of imazapic (A), imazapyr (B), rimsulfuron (C), and sulfosulfuron (D) on AHAS activity of M82 and HRT1 tomato plants. Data were computed by nonlinear regression using Sigma-Plot v. 11.01. (A) $Y = y_0 + \frac{a}{1+\left(\frac{x}{x0}\right)^b}$; for M82: $y_o = 2.3$, $a = 100$, $x_0 = 0.47$, $b = 1.24$, $R^2 = 0.98$, $P < 0.0001$; for HRT1: $y_o = -2.05$, $a = 100$, $x_0 = 3.31$, $b = 0.74$, $R^2 = 0.99$, $P < 0.0001$. (B) $Y = \frac{a}{1+\left(\frac{x}{x0}\right)^b}$; for M82: $a = 100$, $x_0 = 2.52$, $b = 0.97$, $R^2 = 0.99$, $P < 0.0001$; for HRT1: $a = 100$, $x_0 = 6.55$, $b = 0.48$, $R^2 = 0.96$, $P < 0.0001$. (C) $Y = \frac{a}{1+\left(\frac{x}{x0}\right)^b}$; for M82: $a = 100$, $x_0 = 0.02$, $b = 1.3$, $R^2 = 0.99$, $P < 0.0001$; for HRT1: $a = 100$, $x_0 = 0.04$, $b = 0.62$, $R^2 = 0.95$, $P < 0.0001$. (D) $Y = y_o + \frac{100}{1+\left(\frac{x}{x0}\right)^b}$; for M82: $y_o = 1.72$, $a = 100$, $x_0 = 0.0008$, $b = 0.99$, $R^2 = 0.98$, $P < 0.0001$; for HRT1: $y_o = 0.1$, $a = 100$, $x_0 = 0.018$, $b = 1.14$, $R^2 = 0.99$, $P < 0.0001$.

was significantly reduced from 106 to 78 nM g^{-1} (Figure 3B–D). A small, nonsignificant reduction (from 136 to 111 nm g^{-1}) was also obtained for leucine content in the leaves of M82 plants after imazapic treatment (data not shown). In HRT1 leaves, there were no significant differences in the total content of branched-chain amino acids (Figure 3B), or in the leucine (data not shown), isoleucine, or valine content (Figure 3C and 3D). A nonsignificant loss in total content of amino acids (2,792 nM g^{-1} compared with 3,698 nM g^{-1} in the control) (Figure 3A) was observed. Branched-chain amino acids also were decreased in AHAS inhibitor–treated pea (Ray 1984), maize (Anderson and Hibberd 1985), and other plants (Zhou et al. 2007). In addition, the levels of free leucine, isoleucine, and valine were significantly decreased in imazethapyr-treated chickpea lines sensitive to this herbicide, but not in resistant lines (Prakash et al. 2017).

Segregation Analysis

Mutation inheritance is important for breeding programs in which the resistance is to be introduced into elite cultivars. Segregation analysis indicated that 16 (21%) out of 75 and 21 (28%) out of 74 F_2 (M82 × HRT1) plants were resistant to imazapic at a rate of 24 g ai ha^{-1} under greenhouse and field conditions, respectively. The resistance segregated 1:3 in the progeny ($\chi^2 = 0.46$, $P = 0.5$), indicating that resistance to imidazolinones in line HRT1 is due to a single recessive gene. Although AHAS resistance segregates as a single semi-dominant allele in many plant species, such as chickpea (Thompson and Tar'an 2014), canola (Swanson et al. 1988), soybean (Sebastian et al. 1989), sunflower (Sala et al. 2008), wheat (Pozniak and Hucl 2004), sorghum [*Sorghum bicolor* (L.) Moench] (Tesso et al. 2011), and maize (Harms et al. 1990; Newhouse et al. 1991), in other soybean mutants, it segregates, as in our case, as as a single recessive gene (Sebastian and Chaleff 1987). Recessive inheritance is advantageous when transferring the resistance trait to target plants, because it is very easy to screen for this trait, and all resistant plants are homozygous. In contrast, dominant inheritance has the advantage of producing inbred seeds, because the trait only needs to be passed on to one of the hybrid parents.

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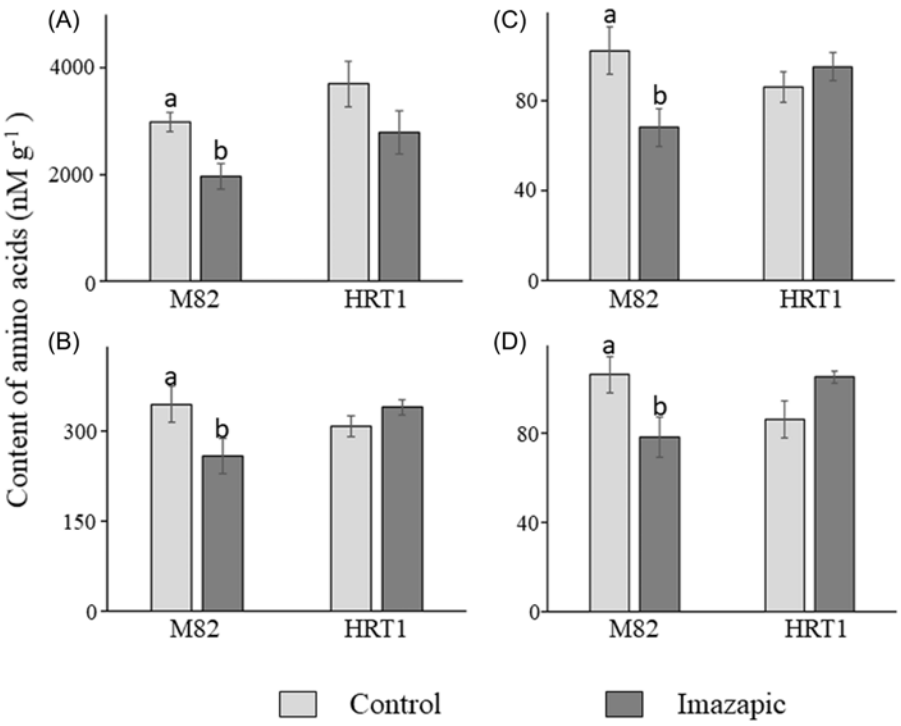

Figure 3. Influence of imazapic treatment on the amino acid content in M82 and HRT1 tomato plant leaves. M82 and HRT1 plants were sprayed with imazapic at a rate of 14.4 g ai ha^{-1}. After 3 wk, leaf samples of treated and nontreated plants were taken for analysis of total amino acids (A), total branched-chain amino acids (B), isoleucine (C), and valine (D). Vertical lines present standard error of the mean (SEM); different letters indicate significant differences between control and imazapic-treated plants of the same line according to Student's *t*-test.

Mutation Changes AHAS1 Structure

The ligand–protein contact server (LPC software; Sobolev et al. 2005) analyzes and visualizes atomic interactions within a protein or protein complex, providing characteristics for every atom–atom contact (atom properties, distance, and contact area). We used this software to examine the microenvironment of the mutated residues. The model of *Arabidopsis* AHAS1 in complex with the imidazolinone imazaquin (IQ) revealed that IQ blocks the active channel of the enzyme formed by the interface of two catalytic monomers (Figure 4A). This corresponds well with work done by McCourt et al. (2006), who presented the first 3D structure of *Arabidopsis thaliana* AHAS in complex with the imidazolinone IQ. Ligand–protein contact analysis showed that the closest distance from Ala-205 to IQ is between the hydrophobic CB atom of Ala-205 and the hydrophilic OC atom of IQ (Figure 4B). This distance is large, and the repulsion is very small. Replacement of Ala-205 by the larger hydrophobic residue Val-205 results in a closer distance to the hydrophilic OC atom of IQ, thereby increasing repulsion and making herbicide binding more difficult (Figure 4). This orientation of Val-205 is due to repulsion from the hydrophilic atom of Thr-203. In this orientation, the distance between the hydrophobic C atom of Val-205 and the hydrophilic O atom of IQ is 3.6 Å. This is in agreement with Jain and Tar'an (2014), who proposed that the presence of Ala-205 in the active site allows for imazamox binding, whereas the presence of valine at the same position disrupts this binding. Moreover, in that study, partially hydrophobic cluster analysis showed that the presence of the more hydrophobic residue (valine) instead of alanine results in a conformational change at the protein interface, modifying the herbicide-binding site (Jain and Tar'an 2014). Thus, the enzyme

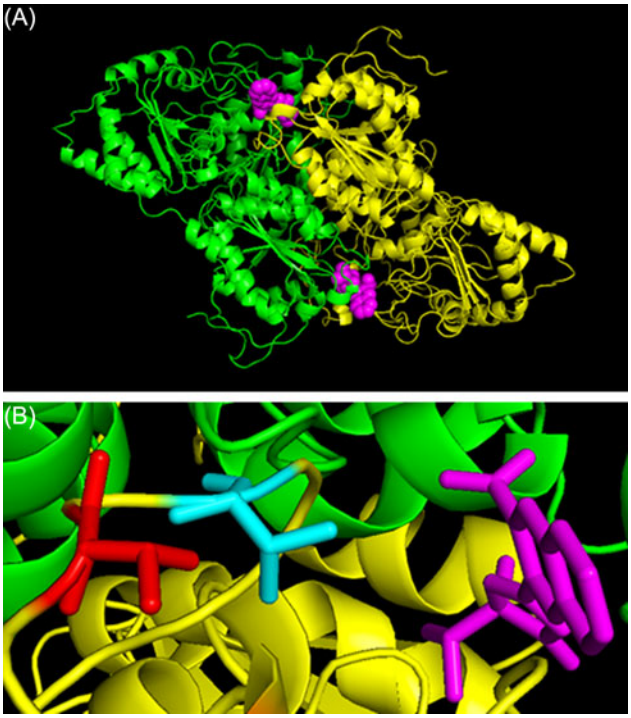

Figure 4. Protein–ligand complex of AHAS1 from *Arabidopsis* with imazaquin (IQ; PDB entry1Z8N). (A) Two molecules of IQ (purple) block the active channels in the AHAS protein dimer (yellow and green). (B) IQ molecule interaction with amino acid residues of the enzyme. Purple, IQ; blue, valine in position 205; red – Thr-203. The orientation of valine is due to repulsion from the hydrophilic O atom of Thr-203. In this orientation, the distance between the hydrophobic C atom of the valine and hydrophilic atom O of IQ is 3.6 Å.

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becomes inaccessible to imidazolinone herbicides, and branched-chain amino acid starvation is prevented (Figure 2 A and B).

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Competing interests. The authors declare no conflicts of interest.

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