Theor Appl Genet (2014) 127:1583–1591
DOI 10.1007/s00122-014-2320-0

ORIGINAL PAPER

Genetic characterization of the *acetohydroxyacid synthase* (*AHAS*) gene responsible for resistance to imidazolinone in chickpea (*Cicer arietinum* L.)

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Received: 17 February 2014 / Accepted: 24 April 2014 / Published online: 13 May 2014

Abstract
Key message **A point mutation in the *AHAS1* gene leading to resistance to imidazolinone in chickpea was identified. The resistance is inherited as a single gene. A KASP marker targeting the mutation was developed.**
Abstract Weed control in chickpea (*Cicer arietinum* L.) is challenging due to poor crop competition ability and limited herbicide options. A chickpea genotype with resistance to imidazolinone (IMI) herbicides has been identified, but the genetic inheritance and the mechanism were unknown. In many plant species, resistance to IMI is caused by point mutation(s) in the *acetohydroxyacid synthase* (*AHAS*) gene resulting in an amino acid substitution preventing herbicide attachment to the molecule. The main objective of this research was to characterize the resistance to IMI herbicides in chickpea. Two homologous *AHAS* genes namely *AHAS1* and *AHAS2* sharing 80 % amino acid sequence similarity were identified in the chickpea genome. Cluster analysis indicated independent grouping of *AHAS1* and *AHAS2* across legume species. A point mutation in the *AHAS1* gene at C675 to T675 resulting in an amino acid substitution from Ala205 to Val205 confers the resistance to IMI in chickpea. A KASP marker targeting the point mutation was developed and effectively predicted the response to IMI herbicides in a recombinant inbred (RI) population of chickpea. The RI population was used in molecular mapping where the major locus for the reaction to IMI herbicide was mapped to chromosome 5. Segregation analysis across an F_2 population and RI population demonstrated that the resistance is inherited as a single gene in a semi-dominant fashion. The simple genetic inheritance and the availability of KASP marker generated in this study would speed up development of chickpea varieties with resistance to IMI herbicides.

Communicated by B. Diers.

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Introduction

Chickpea (*Cicer arietinum* L.) is a relatively new pulse crop on the Canadian Prairies. In western Canada, chickpea is mainly grown on the Brown and Dark Brown soil zones of south-western Saskatchewan (Baker et al. 1996; Padbury et al. 2002; Yadav 2007). Agronomic issues facing chickpea growers in the region include *Ascochyta* blight disease, late maturity and weed pressure. Weed management for chickpea production in the region involves pre-season weed burn-offs using glyphosate or 2-4, D (Baker et al. 1996; Yadav 2007), followed by pre-emergence (sulfentrazone) and post-emergence (metribuzin) herbicide applications. Chickpea crops are often injured by soil residual activity of past herbicide application such as the imidazolinone (IMI) herbicide imazethapyr which can result in yield reduction (Süzer and Büyük 2010; Taran et al. 2013). Also, post-emergent application of metribuzin often causes leaf burn and stand thinning (Taran et al. 2013). Currently, IMI herbicides are registered for use on non-pulse crops such as barley, spring wheat, sunflower, oats, oilseed mustard, canola and alfalfa and pulse crops including lentil, field pea, soybean and dry bean (Saskatchewan Ministry of Agriculture 2013). Benefits of using IMI herbicides include low environmental impact, control of broadleaf weeds and low herbicide dose per hectare (Weed Science Society of America 2007). Development of IMI herbicide-resistant

chickpea varieties will provide more herbicide options for post-emergence weed control and can minimize yield loss due to weed pressure (Kantar et al. 1999; Taran et al. 2010).

In many plant species, resistance to IMI herbicides is the result of a point mutation in the *acetohydroxyacid synthase* (AHAS) also known as *acetolactate synthase* (ALS) (previously classified at E.C. 4.1.3.18 now E.C. 2.2.1.6) gene causing an amino acid substitution (Tan et al. 2005). Mutations in this gene affect key herbicide-binding sites, preventing IMI herbicides from binding and inhibiting *acetohydroxyacid synthase* enzyme activity (Muhitch et al. 1987; McCourt et al. 2006; Duggleby and Pang 2000). Many point mutations in the *AHAS* gene causing resistance to IMI and sulfonylurea (SU) herbicides have been identified (Duggleby et al. 2003; Tan et al. 2005; Beckie and Tardif 2012). Most of the mutations and amino acid substitutions occur at Ala122, Pro197, Ala205, Trp574, and Ser653 (Tan et al. 2005; Heap 2013). Li et al. (2008) reported that an Ala122 mutation on chromosome 6D in wheat (*Triticum aestivum* L.) is responsible for the IMI resistance. A study on wild radish (*Raphanus raphanistrum*) in Australia showed that an Ala122 mutation in *AHAS* resulted in SU, triazolopyrimidine (TP) and IMI cross resistance (Han et al. 2012). Sunflower (*Helianthus spp*), Redroot Pigweed (*Amaranthus retroflexus*) and Eastern Black Nightshade (*Solanum ptychanthum*) have an Ala205 toVal205 mutation that is consistent with IMI herbicide resistance (White et al. 2003; McNaughton et al. 2005; Ashigh and Tardif 2007). In barley (*Hordeum vulgare* L), IMI herbicide resistance was caused by an *AHAS* point mutation from G to A at nucleotide position 1742 (Lee et al. 2011). A Ser to Asn substitution was also characterized in wheat (Pozniak and Hucl 2004). A recent report showed that a change from Pro197 to Ser197 confers the resistance to SU herbicides in soybean (Ghio et al. 2013). Segregation studies in various plant species demonstrated that the resistance to IMI herbicide is monogenic with semi-dominant to dominant gene action (Haughn and Somerville 1986; Wright and Penner 1998; Pozniak and Hucl 2004; Oldach et al. 2008; Lee et al. 2011).

Single nucleotide polymorphism (SNP) markers are gaining popularity because of automation potential, biallelic variation, high abundance in the genome and no need for gel electrophoresis (Rafalski 2002; Ganal et al. 2009). In addition, SNP genotyping platforms like the Illumina GoldenGate® assay (Illumina, San Diego, CA, USA) can be used to quickly develop a molecular map (Rafalski 2002; Hiremath et al. 2012) and to identify the location of the gene responsible for the traits of interest in the plant genome. If the resistance to IMI herbicide in chickpea is the result of a point mutation in the *AHAS* gene, an allele-specific SNP marker could be developed and used for marker-assisted selection, increasing selection efficiency in developing resistant varieties.

Resistance to IMI herbicides has been identified in chickpea (Taran et al. 2010); however, the genetic inheritance and the mechanism leading to the resistance are unknown. Identifying the genetic factor and the mode of inheritance will help in understanding the IMI herbicide resistance mechanism in chickpea and can aid in the selection process to develop herbicide-resistant varieties. The objectives of the present study were (1) to sequence the *AHAS* gene in IMI resistant and IMI susceptible chickpeas and develop allele-specific SNP markers targeting the point mutation causing IMI resistance; (2) to examine the synteny of the *AHAS* gene across pulse crops and other species; (3) to examine the inheritance of the resistance using F_2 and recombinant inbred lines (RILs) segregating for reaction to IMI herbicide; (4) to test the usefulness of the KASP SNP marker for selection of IMI-resistant chickpea progeny, and (5) to map the location of the AHAS gene in the chickpea genome.

Materials and methods

Preparation of plant material was conducted similarly across all research components. Sunshine mix #4 (Sun Grow, Seba Beach, AB) was used as growth medium, but washed using warm water 4–5 times and allowed to drain for a minimum of 2 h before seeding. Desi-type seeds were scarified using tweezers 24 h before sowing. Seeds were also treated using a mixture of fludioxonil, metalaxyl-M S-isomer and thiabendazole (Apron FL®, Syngenta Canada Inc.) to prevent root rot. Plants were grown in the greenhouse with the following conditions 21 °C average air temperature, 21 integrated photosynthetic radiation and 44.0 % relative humidity in winter, and 26 °C average air temperature, 52 integrated photosynthetic radiation, and 80 % relative humidity in summer. Growth chambers at the College of Agriculture and Bioresources were used in herbicide screening experiments and RIL generation advancement. The growth chamber conditions were maintained at 24 °C/14 h day and a 16 °C/10 h night.

PCR and sequencing preparation

Initial *AHAS* gene sequencing was carried out using primers designed from the *Cicer arietinum* (ICC4958) transcriptome database (Garg et al. 2011). The Primer3 online program (www. primer tool; Whitehead Institute for Biomedical Research, 1998) was used to design primers to amplify *AHAS* in three segments, each about 700–800 bp. Initial analysis of the *AHAS* sequences across IMI susceptible and resistant chickpea genotypes did not show any mutation consistent with the herbicide resistance. The

consensus sequence hereafter named as *AHAS2* was not included in this report.

The *AHAS2* consensus sequence was then used as query to search for the homologous *AHAS* gene in the CDC Frontier draft genome sequence (Varshney et al. 2013). The analysis identified a second copy as *AHAS1* (translated amino acid 80 % similar with *AHAS2*). The CDC Frontier *AHAS1* sequence was then used to design primers to sequence *AHAS1* in IMI resistant and IMI susceptible chickpea genotypes. The following is the sequence information for each primer pair to amplify the *AHAS1* gene: (1) Ca—AHAS1-33 (5′-CGCATTACCATCDCACDCAC-3′; forward), (2) Ca—AHAS1-1053 (5′-CTAGGTAGTTAC-CCTGTTGGAGGAG-3′; reverse), (3) Ca—AHAS1-696 (5′-AGATCCATCDCAAAGCATAACTACC-3′; forward), (4) Ca—AHAS1-1495 (5′-CTAACAATAGCATCDCCAT TTGTCA-3′; reverse), (5) Ca—AHAS1-1173 (5′-GATG ATCGTGTAACTGGGAAATTAG-3′; forward), and (6) Ca—AHAS1-2289 (5′-TCDCTTCAACCTGAATCTCDC-TACA-3′, reverse). These primers were used in polymerase chain reaction (PCR) to amplify the *AHAS1* fragments from IMI susceptible and IMI-resistant chickpea genotypes. The PCR components for a 25 µl single reaction were: 4.0 µl of 10 ng μl^{-1} genomic DNA template, 1.0 µl of 10 µM each primer, 2.5 µl 10× buffer, 2.5 µl of 15 mM MgCl, 0.5 µl of 10 mM dNTP, 0.2 µl (1 unit) Genscript Taq polymerase, and 13.3 µl of autoclaved distilled water. The samples were amplified using a BIO-RAD-C1000TM or PTC-100® thermocycler with the following programs: step 1—2 min at 95 °C initial denaturation, step 2—30 s at 94 °C denaturation, step 3—1 min at 60 °C annealing, step 4—1.5 min at 72 °C extension, step 5—return to step 2 for 34 additional cycles, step 6—10 min at 72 °C final extension, and step 7—8 °C until samples removed from thermocycler.

PCR products were mixed with GenScript GelRed™ loading dye then separated and inspected on a 1.5 % agarose gel electrophoresis with Tris–acetate (TAE) buffer. The QIAGEN® QIAquick® Gel extraction kit (QIAGEN Inc. Mississauga, ON) was used to extract and purify DNA of the correct size (about 800–1,000 bp). Eluded DNA was sent for Sanger sequencing at the National Research Council Canada on the University of Saskatchewan Campus (Saskatoon, Saskatchewan). Sequencher® 5.0 software (Gene Codes Corporation, Ann Arbor, MI United States) was used to compare the sequence data and to identify the SNPs.

Cluster analysis

The consensus chickpea *AHAS* sequences were used as query to retrieve the publicly available homologous sequences (NCBI BLAST® online database). The *AHAS1* and *AHAS2* sequences from the CDC Frontier genome were used for cluster analysis in conjunction with the following homologous *AHAS* sequences: *Glycine max* AHAS 2 (XM_003545859.1) 2,146 bp, *Lotus japonicus* AHAS (AK339751.1) 2,197 bp, *Medicago truncatula* (XM_003593479.1) 2,258 bp, *Medicago truncatula* 'Caliph' (EU292216.1) 2,052 bp, *Medicago littoralis* 'Angel' (EU292213.1) 2,165 bp, *Phaseolus vulgaris* 'Olathe' (GQ466185.1) 1,947 bp, *Helianthus annuus* AHAS1 (AY541451.1) 1,968 bp, *Helianthus annuus* AHAS2 (AY541457.1) 1,947 bp, *Sinapis arvensis* AHAS1-R (AY954041.1) 380 bp, and *Sinapis arvensis* AHAS1-R (AY954042.1) 380 bp.

Molecular evolutionary genetics analysis (MEGA) (Tamura et al. 2011) software was used for multiple sequence alignment, cluster analysis, and phylogenetic tree construction. ClustalW in MEGA was used to conduct the initial multi-sequence alignment. A phylogenetic tree was computed using the Neighbor-joining algorithm with Bootstrapping of 500 re-sample data sets.

Inheritance study

An F_2 population (CDC Leader [IMI susceptible] × ICCX860047-9[IMI resistant]) and $F_{7:8}$ RIL population (CDC 512-51 [IMI susceptible] × ICCX860047-9) segregating for herbicide tolerance were used to test the inheritance of the IMI resistance. In addition, six F_1 plants from the CDC Leader × ICCX860047-9 were also used for herbicide screening. All the seeds were scarified and pregerminated in a petri dish with dampened filter paper for 24–48 h. For the RIL, ten seeds per line were used. The germinated seeds were then transplanted into 26 cm^2 pots filled with Sunshine mix #4 medium (Sun Grow, Seba Beach, AB). When plants were between the 2–6 leaf stage (10–14 days after seeding), a spray cabinet was used for herbicide application with the following settings: even-Spray nozzle 8001 EVS, operated at 240 kPa, spray calibrated to 100 l ha^{-1} (Taran et al. 2010). Plants were sprayed with Solo® (70 % imazamox; BASF Canada) at a rate of 28.91 g Solo®/hectare with water volume of 99 l ha^{-1}. Adjuvant Merge® was used at a rate of 0.5 ml/100 ml solution. Herbicide rating of either resistant or susceptible was taken at 7, 14 and 28 days after herbicide treatment. Desi cultivars CDC Corinne (IMI susceptible) and CDC Cory (IMI resistant) were used as the checks in herbicide ratings. A resistant rating was given if there were no changes in plant performance and morphology. A susceptible plant showed typical herbicide injury symptoms such as severely stunted growth, chlorosis, necrosis and small needle-like leaf development. Plants were classified as intermediate if any morphologically changes were apparent. Symptoms

could include slightly stunted growth, minor chlorosis and increased branching. Phenotypic data from this study were also used to determine the mode of inheritance.

KASP SNP genotyping

The RIL population was also used to test the effectiveness of the KASP SNP marker. Plant samples for DNA sourcing were prepared the same as for the *AHAS* sequencing plant samples. Two of the youngest leaves were harvested for DNA source using a modified CTAB procedure (Doyle and Doyle 1987). DNA was quantified using a FLUOstar Omega Fluorometer (BMG LABTECH Ortenberg, Germany) and diluted to 10 ng μl^{-1}. The KBioscience Allele-Specific PCR Genotyping system (KBioscience Ltd., Hoddesdon, UK) was used to develop and test SNP markers for selecting IMI resistance genotypes in the RIL population. Using chickpea *AHAS1* sequence data primers targeting the point mutation in *AHAS* responsible for IMI resistance were designed using Primer Picker Software offered by KBioscience (http://www.kbioscience.co.uk/primer-picker/). The PCR was done using the forward allele-specific primer: 5′-CGGAGAATGATCGGAACCGATG**T/C**-3′ and a common reverse primer 5′-TTTGTGATGGATCTCGTTACTTCAACGAT-3′. The reaction was run on a StepOnePlus™ real-time PCR system with the following program: step 1—60 °C for 30 s (florescence read), step 2—95 °C for 10 min, step 3—95 °C for 15 s, step 4—60 °C for 1 min (repeat step 3–4, 40×) (florescence read), and step 5—60 °C for 30 s (florescence read). Fluorescence data and SNP calls were made using StepOneTM Software v2.1 (Applied Biosystems, Foster City, CA, USA). The first and final florescence reads were used to call each genotype based on the parental SNP data and data displayed on the allelic discrimination plot generated by the software. This data were compared to the RIL herbicide rating data to determine the effectiveness of the KASP marker in predicting the reaction to the IMI herbicides.

Molecular mapping

The same RIL population was also used for development of a SNP-based molecular map. A modified CTAB procedure was used for DNA extraction of each RIL (Doyle and Doyle 1987). DNA was quantified using a FLUOstar Omega Fluorometer (BMG LABTECH Ortenberg, Germany) and diluted to 50 ng μl^{-1}. Then, 20 µl of 50 ng μl^{-1} DNA from each RIL and parental lines were transferred to a 96-well plate. The National Research Council (Saskatoon, Saskatchewan) carried out the 1536 SNP genotyping designed specifically for chickpea using Illumina GoldenGate® Assay. The Illumina GenomeStudio ver 2010.1 Data Analysis Software (Illumina San Diego, CA USA) was used to analyze the SNP genotyping data. A SNP graph was generated by the software and each SNP was individually inspected and classified as monomorphic, polymorphic, heterozygous or failed. Only polymorphic markers between the two parents were used for molecular mapping. The SNP marker data from the RILs were then sorted into linkage groups using CarthaGène with the minimum logarithm of odds (LOD) threshold of 6.0 with a distance threshold of 0.3 recombination fraction (Institut National de la Recherche Agronomique; de Givry et al. 2005). The Kosambi mapping function was used to create the linkage map (Kosambi 1943) which was then aligned with the consensus map developed using the ICCV96029 × CDC Frontier population (Tar'an et al. 2007; Anbessa et al. 2009) using the MapChart version 2.2.

Results

Sequence analysis

Seven chickpea genotypes were used for sequence analysis. The genotypes included both IMI susceptible (Myles, CDC Frontier, CDC Corinne and CDC Luna) and IMI-resistant (ICCX860047-9, CDC Cory and CDC Alma) cultivars. Both nucleotide and amino acid position numbers are based on *Arabidopsis thaliana* (NCBI reference NM_114714.2) *AHAS* sequence which is 2270 nucleotides or 670 amino acids (aa) long. *Medicago truncatula* 'Caliph' (EU292216.1) and *Medicago littoralis* 'Angel' (EU292213.1) were aligned as legume references. The consensus *AHAS1* sequence is 2,183 bp (658 aa) long with no introns. A point mutation at base pair number 675 from cytosine (C) to thymine (T) that resulted in the amino acid substitution Ala205 to Val205 was found to be consistent with the reaction of the cultivars to IMI herbicides (Fig. 1). This mutation is consistent with other known mutations causing resistance to IMI herbicides (Tan et al. 2005; Beckie and Tardif 2012).

Cluster analysis

The *AHAS1* and *AHAS2* across legume genera (*Cicer, Gycine, Lotus* and *Medicago*) cluster independently (Fig. 2, Clusters A and B). The AHAS genes from other plant species such as *Helianthus annuus, Sinapis arvensis* and *Brassica napus* formed separate clusters (Fig. 2. Clusters C and D). Branching pattern and numbers at nodes indicate levels of bootstrap support based on Neighbor-joining analysis of 500 re-sample data sets.

A

Genotype	Nucleotide Sequence (675)
Myles IMI-S	GGAGAATGATCGGAACCGATG**C**TTTTCAAGAAACCCCCATCGTT
CDC Frontier IMI-S	GGAGAATGATCGGAACCGATG**C**TTTTCAAGAAACCCCCATCGTT
CDC Corinne IMI-S	GGAGAATGATCGGAACCGATG**C**TTTTCAAGAAACCCCCATCGTT
CDC Luna IMI-S	GGAGAATGATCGGAACCGATG**C**TTTTCAAGAAACCCCCATCGTT
ICCX860047-9 IMI-R	GGAGAATGATCGGAACCGATG**T**TTTTCAAGAAACCCCCATCGTT
CDC Cory IMI-R	GGAGAATGATCGGAACCGATG**T**TTTTCAAGAAACCCCCATCGTT
CDC Alma IMI-R	GGAGAATGATCGGAACCGATG**T**TTTTCAAGAAACCCCCATCGTT

B

Genotype	Amino Acid Sequence (205)
Myles IMI-S	MDSIPIIAITGQVPRRMIGTD**A**FQETPIVEVTRSITKHNYLILE
CDC Frontier IMI-S	MDSIPIIAITGQVPRRMIGTD**A**FQETPIVEVTRSITKHNYLILE
CDC Corinne IMI-S	MDSIPIIAITGQVPRRMIGTD**A**FQETPIVEVTRSITKHNYLILE
CDC Luna IMI-S	MDSIPIIAITGQVPRRMIGTD**A**FQETPIVEVTRSITKHNYLILE
ICCX860047-9 IMI-R	MDSIPIIAITGQVPRRMIGTD**V**FQETPIVEVTRSITKHNYLILE
CDC Cory IMI-R	MDSIPIIAITGQVPRRMIGTD**V**FQETPIVEVTRSITKHNYLILE
CDC Alma IMI-R	MDSIPIIAITGQVPRRMIGTD**V**FQETPIVEVTRSITKHNYLILE

C

Species	Amino Acid Sequence (197, 205)
Arabidopsis thaliana	LDSVPLVAITGQV**P**RRMIGTD**A**FQETPIVEVTRSITKHNYLVMD
Medicago truncatula SU-S	MDSVPLIAITGQV**P**RRMIGTD**A**FQETPIVEVTRSITKHNYLILD
Medicago littoralis SU-R	MDSVPLIAITGQV**L**RRMIGTD**A**FQETPIVEVTRSITKHNYLILD
C. arietinum IMI-S	MDSIPIIAITGQV**P**RRMIGTD**A**FQETPIVEVTRSITKHNYLILE
C. arietinum IMI-R	MDSIPIIAITGQV**P**RRMIGTD**V**FQETPIVEVTRSITKHNYLILE

Fig. 1 Multiple alignment of portion of *AHAS1* nucleotide (**a**) and amino acid (**b**) sequences across seven chickpea (*Cicer arietinum*) genotypes. **c** *AHAS1* amino acid sequences alignment across chickpea and model plant species. (Alignment based off of *Arabidopsis thaliana* NCBI reference NM_114714.2). Point mutation leading to the IMI resistant is indicated by an *arrow*. *IMI-R* IMI resistant, *IMI-S* IMI susceptible, *SU-S* sulfonylurea susceptible, *SU-R* sulfonylurea resistant

Inheritance

Reaction of the six F_1 plants from CDC Leader (IMI susceptible) × ICCX860047-9 (IMI resistant) cross showed an intermediate symptom (stunted growth, minor chlorosis and increased branching) indicating a semi-dominant gene action. A total of 88 F_2 plants derived from the CDC Leader × ICCX860047-9 cross segregated for 26 resistant: 42 intermediate: 20 susceptible. Chi-square test indicated that the ratio followed a 1:2:1 ratio ($\chi^2 = 0.71$; $P = 0.61$) for a single gene semi-dominant model. Furthermore, screening of 70 RILs ($F_{7:8}$) from the cross of CDC512-51 (IMI susceptible) × ICCX 860047-9 for their response to IMI herbicide resulted in 40 resistant and 30 susceptible. Chi-square analysis suggested that the segregation followed a 1:1 ratio for a single gene model ($P = 0.232$).

KASP SNP Genotyping

Only 64 RILs of the CDC512-51 × ICCX 860047-9 cross that had good quality DNA plus parental lines were used to test the KASP SNP marker. The parental lines each fell into different clusters. Based on clustering and parental florescence data, SNP genotypes were called as allele A for parental type CDC 512-51 (IMI susceptible) and allele B for parental type ICCX860047-9 (IMI resistant). The SNP genotyping data were then compared to the herbicide rating data. The KASP SNP genotyping platform accurately predicted herbicide response of 63 RILs (Fig. 3). One line could not be grouped into either cluster A or B due to weak signal.

Molecular mapping

Out of 1,536 SNP markers, 530 were polymorphic between CDC 512-51 and ICCX860047-9 with 507 markers used to create the linkage map. These linkage groups correspond to the first seven ICCV96029 × CDC Frontier chromosomes (Varshney et al. 2013). The chromosome 8 is not accounted for. Two linkage groups could not be linked with the ICCV96029xCDC Frontier consensus map due to inadequate marker data. The *AHAS* gene was linked to two SNP markers, Cav1sc310.1p304295 at 6.6 cM and Cav1sc1.1p4940145 at 3.8 cM (Fig. 4). The Cav1sc1.1p4940145 SNP marker was previously mapped to chromosome 5 in the ICCV96029 × CDC Frontier map.

Discussion

The *AHAS* gene has been extensively studied and reviewed in many plant species (Tan et al. 2005). IMI herbicides inhibit the acetohydroxyacid synthase (AHAS) enzyme and resistance is usually the result of a point mutation in the

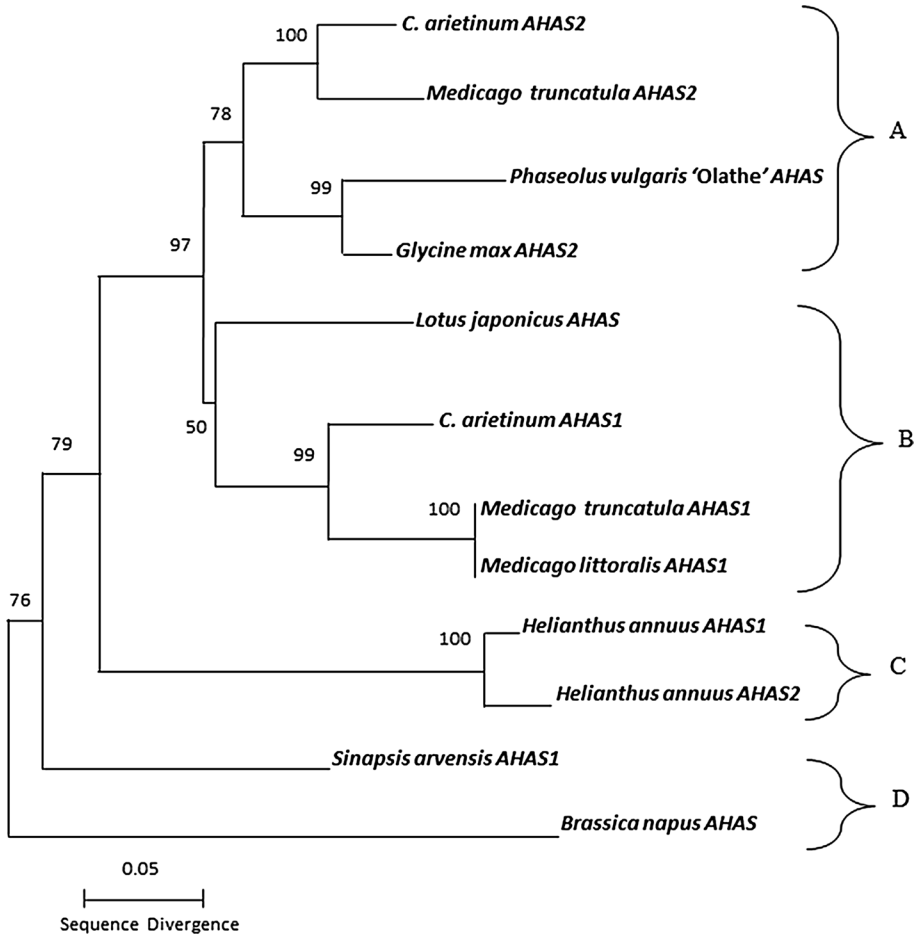

Fig. 2 Phylogenetic tree of *AHAS1* and *AHAS2* across legumes and other plant species using Neighbor-joining method (bootstrap replications = 500). Bootstrap confidence intervals are placed at each node and are expressed as a percentage. Groups of *AHAS* and species are indicated by *different letters* on the *right*. The *AHAS1* cluster in legumes is indicated by *B* and the *AHAS2* cluster is indicated by *A*

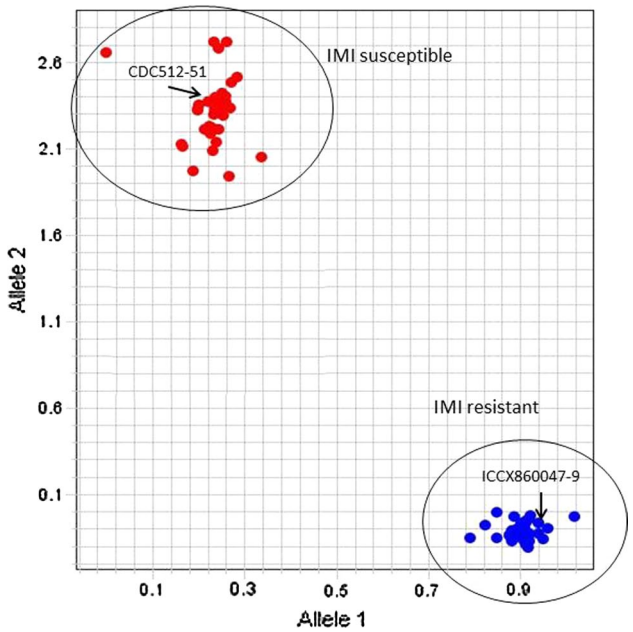

Fig. 3 Allelic discrimination plot of KASP SNP genotyping of $F_{7:8}$ CDC 512-51 × ICCX860047-9 population segregating for resistance to IMI herbicide. Parental type (CDC 512-51 and ICCX860047-9), IMI resistant and IMI susceptible groups are indicated by *circles*

AHAS gene causing amino acid substitution in the AHAS enzyme (Tan et al. 2005; Beckie and Tardif 2012). Point mutations in *AHAS* have been linked to varying degrees and spectrum of resistance to IMI and SU herbicides (Tan et al. 2005). *AHAS* sequences from seven chickpea cultivars were compared. In chickpea *AHAS1*, a C to T mutation at nucleotide #675 resulting in an Ala205 to Val205 substitution confers the resistance to IMI. In Redroot Pigweed (*Amaranthus retroflexus*) and Eastern Black Nightshade (*Solanum Ptychanthum*), an Ala205 substitution also causes IMI resistance (McNaughton et al. 2005; Ashigh and Tardif 2007; Beckie and Tardif 2012). Research in sunflower (*Helianthus annuus*) showed an Ala205 substitution resulted both in IMI resistance and partial SU resistance (Kolkman et al. 2004). McCourt et al. (2006) initially did not identify Ala205 as a binding site, so more structural analysis on the effect of Ala205 substitutions on herbicide binding may be needed. In *Medicago spp.*, a Pro197 to Leu197 resulted in SU resistance (Fig. 1) (Oldach et al. 2008). A Pro197 substitution in the *AHAS* gene results in phenotype with varying levels of resistance to SU, pyrimidinylthiobenzoates (PTB) and triazolopyrimidines (TP) (Haughn et al. 1988; Mourad and King 1992; Beckie

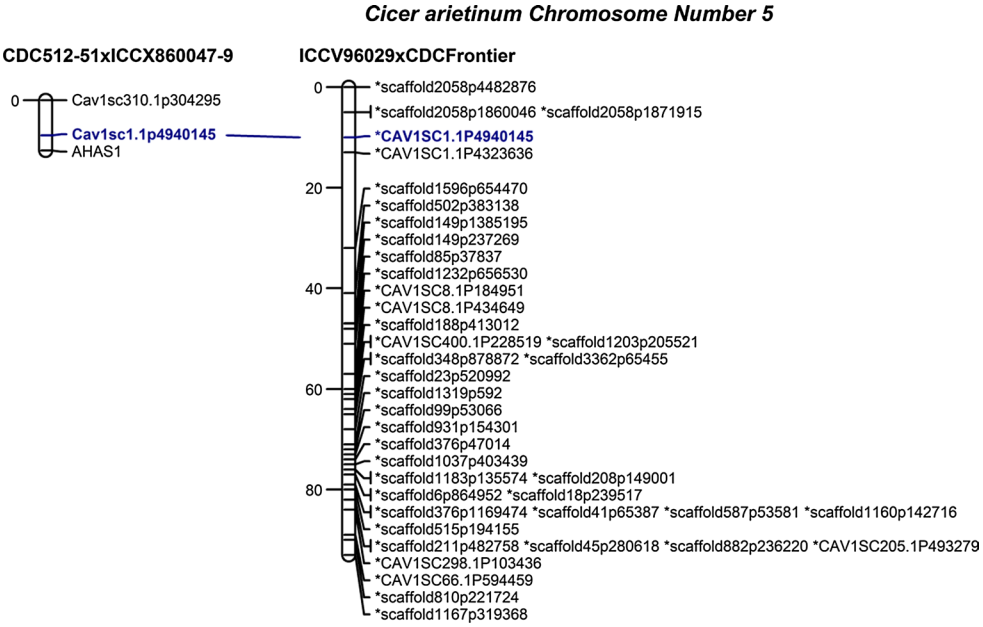

Fig. 4 Alignment of *Cicer arietinum* linkage group 5 of CDC512-51 × ICCX860047-9 RIL population segregating for IMI resistance and Chromosome 5 of ICCV96029 × CDC Frontier consensus map (genetic distance is in cM Kosambi function)

and Tardif 2012), which is consistent with the findings of McCourt et al. (2006) that Pro197 is in direct contact with SU but indirectly with IMI. This suggests that specific resistance to IMI, PTB, sulfonylaminocarbonyltriazolinone (SCT), TP or SU or cross resistance among them is associated with the mutation site(s) within the *AHAS* gene and may not be conserved across different species.

Depending on the species, there may be a single copy or multiple copies of the *AHAS* gene. For example, in *Arabidopsis thaliana*, only *CSR1* codes for AHAS (Haughn and Somerville 1986; Haughn et al. 1988; Manabe et al. 2007). Some species have multiple homologous *AHAS* genes. In *Brassica napus*, *AHAS1* and *AHAS2* are 85 % similar and *AHAS1* and *AHAS3* are 98 % similar (Rutledge et al. 1991). Rutledge et al. (1991) determined that each copy may have originated from each *Brassica napus* ancestor genome. RNase protection assays showed that *AHAS*1 and *AHAS*3 were expressed in all plant tissues, but *AHAS2* was only expressed in mature ovule and immature seed tissue, meaning *AHAS2* may have a specific role in seed development in *Brassica napus* (Ouellet et al. 1992). Sunflower (*Helianthus annuus*) has three homologous *AHAS* genes, *AHAS1* and *AHAS2* are 92 % identical, and *AHAS3* is only 72 % identical to *AHAS1* and 73 % similar to *AHAS2,* respectively (Kolkman et al. 2004). Sunflower *AHAS1* and *AHAS3* were predominantly expressed in leaf tissue, which is logical since AHAS is located in chloroplasts (Miflin 1974; Smith et al. 1989). In chickpea, there are two homologous *AHAS* genes: *AHAS1* and *AHAS2.* Even though the genes are 81 % similar, only mutation in *AHAS1* confers to IMI resistance. To date, the role of multiple *AHAS* genes in chickpea is unclear and additional research may be needed.

Chickpea *AHAS1* and *AHAS2* sequences were compared to other known *AHAS* sequences. Cluster analysis showed that *AHAS1* and *AHAS2* genes were grouped separately, except in non-legume species such as *Sinapis arvens, Helianthus annuus* and *Brassica napus*. This result suggests that the two copies may have different function or are expressed in different organs as reported in *Brassica napus* (Ouellet et al. 1992). Expression and functional analysis of *AHAS1* and *AHAS2* in chickpea is needed to test this hypothesis.

An allele-specific SNP marker (KASP) was developed and tested for its potential use in marker-assisted selection (MAS) for IMI-resistant chickpea. Use of SNP markers has been reviewed and the main benefits include good distribution throughout the genome, no electrophoresis required, low cost, reproducible results, and potential for automation (Syvanen 2005; Xu and Crouch 2008). The KASP marker targeting the point mutation in the *AHAS1* gene was used to screen a chickpea RIL population segregating for herbicide resistance. The marker accurately predicted phenotypic response to IMI herbicides in a RIL population. To confirm the broader use and accuracy of this marker, larger segregating populations might be needed.

This study used 1536 SNPs in the Illumina GoldenGate® genotyping platform to map the location of the IMI resistance gene in chickpea. The map which was developed using the CDC 512-51 × ICCX860047-9 RIL population covered seven out of the eight chickpea chromosomes with the *AHAS1* gene located on chromosome 5. IMI response segregated as a single gene and was linked to two SNP markers namely Cav1sc310.1p304295 and Cav1sc1.1p4940145. However, this linkage group only consisted of these two markers. Low marker coverage may be due to small sample size (70 RIL lines), lack of variation

(besides IMI resistance) between the parents (CDC 512-51 × ICCX860047-9) where both parents are Desi type.

In conclusion, the study revealed that two homologous *AHAS* genes are present in the chickpea genome. The two *AHAS* sequences (*AHAS1* and *AHAS2*) clustered separately in chickpea and across other legume genera. A point mutation in *AHAS1* gene at C675 to T675 resulted in IMI resistance in chickpea. Using this information, an allele-specific KASP marker targeting the point mutation was developed. The availability of this marker could enhance the selection process in the breeding program to develop resistant varieties. Segregation analysis using IMI treated F_1 plants, F_2 and RIL populations showed that the resistance is controlled by a single gene in a semi-dominant fashion. Using the Illumina GoldenGate® SNP genotyping assay, IMI resistance was mapped to chromosome 5 similar to the position of *AHAS1* in the pseudomolecules of CDC Frontier.

Acknowledgments The authors thank Parvaneh Hashemi, Carmen Breitkreutz, Robert Stonehouse, Mauricio Parada, Ambuj Jha and Amit Deokar for their technical support. Funding for this research was provided by the Saskatchewan Pulse Growers (BRE#0906 and BRE#1010).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiments conducted in this research comply with the current laws of Canada.

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