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Characterization of the acetohydroxyacid synthase multigene family in the tetraploide plant *Chenopodium quinoa*

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ABSTRACT

Background: Currently, the technology called Clearfield® is used in the development of crops resistant to herbicides that inhibit the enzyme acetohydroxy acid synthase (*AHAS*, EC 2.2.1.6). *AHAS* is the first enzyme of the biosynthetic pathway that produces the branched-chain of the essential amino acids valine, leucine, and isoleucine. Therefore, multiple copies of the *AHAS* gene might be of interest for breeding programs targeting herbicide resistance. In this work, the characterization of the *AHAS* gene was accomplished for the *Chenopodium quinoa* Regalona-Baer cultivar. Cloning, sequencing, and Southern blotting were conducted to determine the number of gene copies.
Results: The presence of multiple copies of the *AHAS* gene as has been shown previously in several other species is described. Six copies of the *AHAS* gene were confirmed with Southern blot analyses. *CqHAS1* and *CqAHAS2* variants showed the highest homology with *AHAS* mRNA sequences found in the NR Database. A third copy, *CqAHAS3*, shared similar fragments with both *CqAHAS1* and *CqAHAS2*, suggesting duplication through homeologous chromosomes pairing.
Conclusions: The presence of multiple copies of the gene *AHAS* shows that gene duplication is a common feature in polyploid species during evolution. In addition, to our knowledge, this is the first report of the interaction of sub-genomes in quinoa.

1. Introduction

Quinoa (*Chenopodium quinoa* Willd) is an allotetraploid plant ($2n = 4\times = 36$) [1], that is native to the Andean region of South America, and has been cultivated for thousands of years [2]. Recent international attention given to quinoa is centered on its unusually high nutritional value [3]. Compared with cereals, quinoa's protein content is higher (14 to 16%) and provides an ideal balance of essential amino acids for human consumption [4]. Despite these properties, there has been a lack of research and support for quinoa to the point that it is considered a neglected crop [5]. The quinoa crop in northern Chile is cultivated primarily by indigenous Aymara in the Chilean Altiplano; nevertheless, the cultivation of quinoa extends to the south-central zone of Chile in a fragmented pattern. Chilean quinoa is characterized by a broad range of morphological diversity that likely resulted from artificial selection, natural selection, and genetic drift as landraces were introduced to south-central Chile via the trade and migration of indigenous peoples [6].

The productive potential of the Regalona-Baer cultivar (that belongs to a group of coastal ecotypes) in Chile is very high and comparable to the yield of rice (6.2 t ha^{-1}) [7]. This variety of quinoa produces yields of 3 t ha^{-1} and can even produce over 8 t ha^{-1} in small plots [8]. The world production of quinoa has increased over the last ten years mostly due to a rise in cultivated land and not by improvements in production per hectare [7]. The huge gap between the real and the potential yield of this crop generates a spiral of negative effects that restrict the expansion of this valuable crop. Probably, the most important constraint on the industrialized production of crops is weed control [9]. This is also true for quinoa production where weed control has a major impact and is one of the main factors that has limited quinoa expansion [10]. Weeds may have a major impact on seed yield. For instance, it has been shown that the yield may be tripled if grass weeds are controlled [11]. After quick emergence, further development is slow, and quinoa may be over grown by weeds [12]. So far, there are no registered herbicides for quinoa, and in addition, *C. quinoa* is a crop species for which problem weeds include closely

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related members of the family Amaranthaceae [13], making classical herbicide selection difficult. The development of herbicide-resistant crops has resulted in significant changes in agronomic practices, one of which is the adoption of effective, simple, low-risk, crop-production systems with less dependency on tillage and lower energy requirements [14].

In recent years, different companies have conducted extensive research and technological development through public–private partnerships in order to develop crops resistant to herbicides. Commercially, one of the products developed using this approach is called Clearfield®. By 2004, five imidazolinone-tolerant crops, in combination with four imidazolinone herbicides, have been commercialized in different regions of the world and make up what is called the Clearfield® production system [15]. This technology is based on the resistance to herbicides that inhibit the enzyme acetohydroxy acid synthase (AHAS, EC 2.2.1.6). AHAS, is the first enzyme necessary for the biosynthetic pathway that produces the branched-chain essential amino acids valine, leucine, and isoleucine [16,17]. AHAS is also the common target site of five herbicide chemical groups: sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl-thiobenzoates (PTB) and sulfonyl-aminocarbonyl-triazolinone (SCT). Inhibition of AHAS starves plants of valine, leucine, and isoleucine, which leads to plant death [18,19,20].

Depending on the ploidy level, individuals may have a variable number of AHAS gene copies and these copies can be more complex than those found in diploid organisms [21,22]. Multiple AHAS genes have been reported in many plant species belonging to different genera and having different ploidy [23,24]. Regardless of the high nutritional and economic potential of C. quinoa, there are few studies related to increasing the cultivation and yield of this crop. The first step to increase production would be to achieve efficient weed control. In light of this, the aim of this study is to characterize the gene family encoding the AHAS enzyme in the quinoa Regalona-Baer cultivar to provide the basic information needed for a herbicide resistant breeding program.

2. Materials and methods

2.1. Plant material and DNA extraction

For the extraction of genomic DNA, a unique assortment of quinoa cultivars registered in Chile (Regalona-Baer) was used. Seeds were sown in 300 ml pots with a mixture of soil/sand and were grown using the following greenhouse conditions: 20/12°C day/night (± 4°C) with a light regime of 14/10 h day/night and 900 µmol m^{-2} s^{-1} light intensity. Leaf tissue was harvested when plants had four true leaves and DNA extraction was performed using the CTAB modified protocol [25]. DNA was quantified by absorbance at 260/280 nm [26]. Extracts were diluted in distilled water (20 ng µl^{-1}) and stored at -20°C for future analysis.

2.2. AHAS gene discovery

To select and design primers, existing information from the NCBI GenBank database on the closely related genus Amaranthus, Bassia and Salsola was used. For sequence alignment, Amaranthus hypochondriacus, Amaranthus tuberculatus, Amaranthus retroflexus, Amaranthus powellii, Bassia scoparia and Salsola tragus were used. Conserved regions were identified and primers were designed using the Primer3 software [27] (Table 1). The PCR mix consisted of 2 µl of total DNA (20 ng µl^{-1}), 2 µl of each primer (10 ρM), 4 µl of MgCl$_2$ (25 mM), 2 µl of each dNTP (2.5 mM), 2 units of Platinum Taq DNA polymerase and 5 µl of 10× buffer supplied with the enzyme, in a final volume of 50 µl. The amplification protocol consisted of 3 min incubation at 94°C, 34 cycles of 35 s at 94°C, 45 s at x°C, and 105 s at

72°C, and a final extension of 7 min at 72°C. x°C represents the annealing temperature for each pair of primers used (Table 1 and Table 2).

Amplified products were analyzed by gel electrophoresis using a 1.2% agarose gel prepared with 1 × TAE buffer and ethidium bromide 0.5 µg ml^{-1}. The gel was run for 30 min at 100 V. DNA bands were visualized under UV light, compared with a molecular weight marker, and photographed. Fragments of interest were then sequenced by Macrogen, Korea. Confirmation of identity and consensus sequence identification was performed with nucleotide Blast and BlastX of NCBI. The transit peptide of AHAS was identified using ChloroP and TargetP 1.1 [28].

To determine the number of gene copies, the kit pGEMT Easy Vector kit (Promega) was used for cloning the fragment B (499 bp) (Table 2) following all manufacturer's instructions. This fragment was selected because it showed the clearest chromatograms and contains three of the five conserved protein domains. Thirty colonies positive for each transformation were selected and amplified using primers SP6 and T7. Following this, sequencing was conducted for both strand directions; this stage was made by Macrogen, Korea. To infer the phylogenetic relationship of the sequenced genes, phylogenetic trees were constructed using the program MEGA with the statistical methods of maximum parsimony and Bootstrapping using 1000 replicates [29].

Furthermore, a Southern blot analysis was done using the protocol of Sambrook [30]. The FastPCR program was used to search enzymes that do not cut the sequence of the probe used (BspTI and SspI), and as control, enzymes that cut one copy (Copy 2) (Mph1103I), and

Table 1
Primers used for the amplification of the AHAS gene sequence.

Primer	Nucleotide sequence (5′–3′)	Genus
F10	CCTAAACCTAAACCTCCTTC	Amaranthus
F1	TTTTGTTTCCCGATTTAGCCC	Amaranthus
F3	ATTCCTCCGCAATACGCCATT	Amaranthus
R4	AATCAAAACAGGTCCAGGTC	Amaranthus
R1	CCTACAAAAAGCTTCTCCTCTATAAG	Amaranthus
RUTH-F-1C	CKGGCCGTGTKGGTGTTTG	Salsola
RUTH-R-3B	AACTTGTTCTTCCATCACCTTCG	Salsola
B1-F	ATGGCGTCTACTTGTGCAAATCC	Bassia
CHALSF1	GCGTCTACTTGTKCAAAYC	Chenopodium
CHALSF4	GACCTGGACCTGTTTTGATT	Chenopodium
CHALS R1	CAAAGTAACAAGCAACATRAMAAC	Chenopodium
ALS1FB*	ATCACCCCTTCTCTTCTTCAA	Chenopodium
ALS1RD*	CAACAACAAACTAACCTAAAGCA	Chenopodium
ALSGR1*	CATCAAACCTAACCCCGAAA	Chenopodium
ALS2RD*	AGTAGTAGCAAGCAGCATGTG	Chenopodium
ALSGF2*	TTTCGGGGTTAGGTTTGATG	Chenopodium
ALS2F*	ACAAAAATAAACCCTACTCCGTA	Chenopodium

* Copy-specific amplification.

Table 2
Results of the combinations of primers used for amplification of the AHAS gene sequence.

Combination	Amplification
F10–R4	Unspecific
F1–R4 (Fragment B)	Positive at 58°C
F1–CHALSR1	Unspecific
CHALSF1–R4 (Fragment A)	Positive to 57°C
F3–CHALSR1	Unspecific
CHALSF4–R1	Unspecific
CHALSF4–CHALSR1	Unspecific
B1-F–CHALSR1	Unspecific
B1-F–R4	Unspecific
F1–RUTH-R-3B (Fragment E)	Positive at 58°C
CHALSF4–RUTH-R-3B (Fragment D)	Positive at 57°C
RUTH-F-1C–RUTH-R-3B (Fragment C)	Positive at 60°C
*ALS1FB–ALSGR1	Positive at 58°C
*ALS2F–ALSGR1	Positive at 58°C
*ALSGF2–ALS1RD	Positive at 58°C
*ALSGF2–ALS2RD	Positive at 58°C

* Copy-specific amplification.

enzymes that cut all copies (EcoRI). For hybridization and detection, the DecaLabel Biotin Labeling Kit™ DNA Detection Kit and Chromogenic Biotin were used following the instructions of the manufacturer. The analysis of bands was performed with the UN-SCAN-IT gel 6.1 software; and DNA bands were visualized under UV light, compared with a molecular weight marker, and photographed.

The new sequences registered for quinoa were compared with quinoa *AHAS* mRNA obtained from aerial and root tissue of seedlings with 36 days of growth, that have been used for analyzing the transcriptome of quinoa (#2238, KM233690 and #2237, KM233691; Ecotype R49; Morales, Zurita and Silva, unpublished results), using the program MEGA with the statistical methods of maximum parsimony and Bootstrapping using 1000 replicates [29].

3. Results

3.1. Isolation and identification of AHAS in C. quinoa

Five primer combinations were used for direct sequencing since they contain the region spanning the transit peptide and the domains C, A, D, B and E (Table 2). The best amplifications were obtained with fragments B and D. The PCR fragments in total contained 1992 bp, not including the start region and the stop codon.

The confirmation of identity of the new fragment of quinoa using the BLASTN [31] from NCBI, exhibited E-values of 0,0; and an 87% of identity with *B. scoparia*, 86% with *S. tragus*, and 84% with *A. powellii*, *A. retroflexus* and *A. tuberculatus*. Analyses with ChloroP and TargetP 1.1 show that the transit peptide has an estimated size of 50 amino acids, with a score of 0.989 for prediction of localization in the chloroplast.

The complete *AHAS* gene sequence has 2001 bp and does not contain introns; with a total of 667 amino acids including the start and stop codons. The phylogeny made using maximum parsimony grouped the *AHAS* sequence of *C. quinoa* within the subfamily Chenopodiaceae, family Amaranthaceae (Fig. 1).

3.2. Sequence variation in the AHAS gene of C. quinoa

Analysis of the chromatograms obtained from sequencing performed by Macrogen revealed double peaks in the same position in the chromatogram, suggesting the presence of more than one copy of the *AHAS* gene (data not shown). The presence of the *AHAS* gene family in polyploid quinoa was confirmed by cloning and sequencing

fragment B which indicated the presence of six copies: *CqAHAS1*, *CqAHAS2*, *CqAHAS3*, *CqAHAS4*, *CqAHAS5* and *CqAHAS6* (Fig. 2). These six copies were validated using a Southern blot (Fig. 3).

The copies of the *AHAS* gene were compared with mRNA sequences #2238, KM233690 and #2237, KM233691. The analysis of the hypothetical phylogenetic relationships *AHAS* gene copies in *C. quinoa* inferred by maximum parsimony formed two well-delineated groups: copy 1 and copy 4 had 99.7 and 99.5% identity with sequence #2238, respectively. While, copies 2, 5, and 6 showed 99.7, 99.5, and 99.3% identity with sequence #2237, respectively (Fig. 2). The *CqAHAS3* copy is mainly separated from the two groups and most likely lies between the two groups.

In addition, we isolated the complete sequence of the most representative (*CqAHAS1*, KM253767 and *CqAHAS2*, KM253768) copies of the Regalona-Baer variety. Based on these sequences, we designed specific primers for each gene copy (Table 1). Expected PCR products were obtained and direct sequencing was possible and feasible. This procedure will allow us to conduct, in the future, TILLING and ECO-TILLING analyses for the identification of putative point mutations and/or early selection based on an allele-specific analysis of natural and mutant populations of quinoa [32].

Interestingly, when looking at the results of the *CqAHAS3* copy in the grouping, we analyzed the nucleotide sequence, finding an unexpected result: the initial part of the segment (133 bp) was similar to *CqAHAS1*, while the rest of the segment (348 bp) was more similar to *CqAHAS2* (Fig. 4).

4. Discussion

The 2001 bp sequencing encoding the *AHAS* gene in *C. quinoa* is similar to that of *Bassia* while it differs from *Amaranthus*. The *AHAS* gene in *Amaranthus* is 2010 bp long, and the differences in the gene size could be due to polymorphic indels in the C- and N-terminal regions of the protein [17]. Nevertheless, these terminal regions are involved in intracellular traffic rather than in the catalytic activity of the enzyme [17].

Despite these mentioned differences, the 50-aa transit peptide and localization cellular of *C. quinoa* is similar to that of *A. tuberculatus* [33], but differs from the three *AHAS* copies of *Brassica napus* [34] and *Arabidopsis thaliana* [35]. Furthermore, the *AHAS* gene sequence of *C. quinoa*, with a total of 667 amino acids without the presence of introns, is similar to that of many monocots and dicots [36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52]. Exceptional
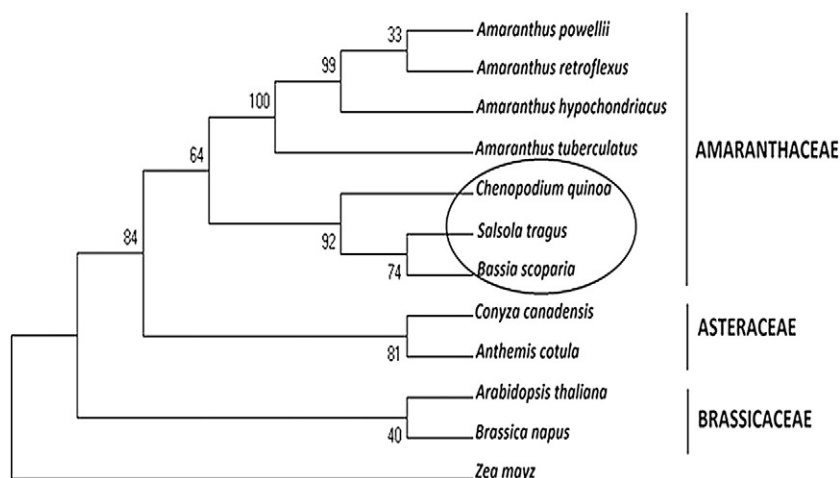
Fig. 1. Hypothetical phylogenetic relationships of plants inferred by maximum parsimony. *AHAS* sequences including the new sequence of *Chenopodium quinoa* (*CqAHAS1*) inferred by maximum parsimony. The oval shows the Chenopodiaceae subfamily members. The numbers at the nodes represent bootstrap values (1000 replicates). *Amaranthus powellii*, *Amaranthus retroflexus*, *Amaranthus hypochondriacus*, *Amaranthus tuberculatus*, *Salsola tragus*, *Bassia scoparia*, *Conyza canadensis*, *Anthemis cotula*, *Arabidopsis thaliana*, *Brassica napus*, *Zea mays*.
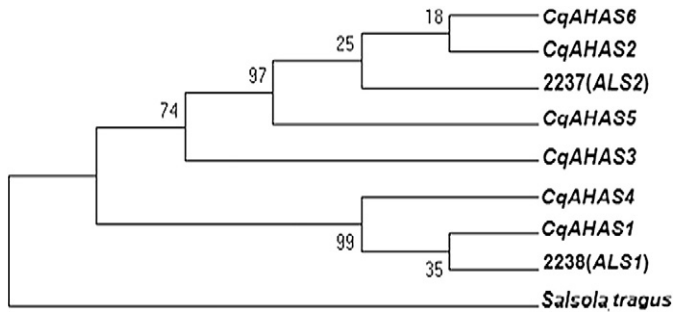
Fig. 2. Hypothetical phylogenetic relationships among *AHAS* gene copies in *Chenopodium quinoa* inferred by maximum parsimony. The numbers at the nodes represent bootstrap values (1000 replicates). *CqAHAS1*, *CqAHAS2*, *CqAHAS3*, *CqAHAS4*, *CqAHAS5*, *CqAHAS6*, 2238, 2237 and *Salsola tragus*.

cases are *Lindernia* spp. [53], *Schoenoplectus juncoides* [54], and *Schoenoplectus mucronatus* [55] whose sequences possess introns and therefore have alternative splicing.

The six variants of the *AHAS* gene found in this study were unexpected for *C. quinoa* as diploid species of the Amaranthaceae family, are thought to have only one copy of this gene [43]. Only the tetraploid species *Salsosa tragus* exhibits two variants [39]. The maximum parsimony phylogenies integrating sequences obtained by cloning (481 bp) and mRNA sequences retrieved from databases, suggest that only *CqAHAS1* and *CqAHAS2* are functional copies. It is possible that each of these two copies derived from the different diploid parental ancestor and underwent gene duplication after hybridization and polyploidization. Regarding the *CqAHAS3* copy, the initial part of the segment (133 bp) was similar to *CqAHAS1* however the rest of the segment (348 bp) was more similar to *CqAHAS2* (Fig. 4). This suggests homeologous chromosome pairing assuming that *CqAHAS1* and *CqAHAS2* come from different diploid ancestors. In fact, Pecinka et al. [56] point out that in polyploids, an increase in the frequency of meiotic recombination (due to pairing and sorting of not only more than two homeologous chromosomes, but also of the homeologous chromosomes during meiosis) could boost the amount of genetic diversity, thereby enhancing the adaptation potential of the organism in challenging natural environments.

The third copy and other variants (4, 5 and 6) of the *AHAS* gene found in *C. quinoa* seem to be silent or might be expressed in a specific tissue. Further gene expression studies are needed to corroborate this. The presence of multiple copies of *AHAS* in the active, silenced, sub-functionalized or neo-functionalized form is well documented in databases particularly for allotetraploid species [21,39,57]. Based on this, polyploids typically show a redistribution and/or increase in the number of loci [58] as a product of gene duplications [59]. The complete sequencing of several eukaryotic genomes shows the importance of duplicated genes [60,61]. In particular, plant genomes contain a high proportion of duplicated genes that are also often highly redundant. In several plants, there are more than one hundred documented paralogous genes [62,63].

The results of this came as part of the complexity of the quinoa tetraploid genome, reflecting, as in other studies, the allotetraploidy in quinoa [64,65,66,67], with functional alleles having been retained at some duplicated loci and at least, some association occurring between homeologous chromosomes, product of erratic multivalent formation at meiosis [64]. Different situation happens with other genes, such as the Salt Overly Sensitive 1 (*SOS1*), where Maughan et al. [68] suggest possible conservation of synteny across the *C. quinoa* sub-genomes across the homeologous *SOS1*.

Multiple copies of the *AHAS* gene are probably not necessary for the growth and development of the species [69]. However, in mutation breeding programs, having multiple copies of *AHAS* genes could have a positive effect on herbicide resistance [22,70,71]. Multiple copies of a gene, product of its polyploidy, bring benefits naturally, without generating gene stacking with transgenic techniques [72].

In this context, higher levels of resistance, due to an increased number of copies of the gene and resistance additive effect [22,71], are desirable to ensure minimal plant injury at herbicide addition rates that are required for adequate weed control [73]. Higher levels of resistance to *AHAS* inhibiting herbicides have been observed in polyploid species when multiple resistance alleles are present [70]. It has been described that alleles conferring imidazolinone tolerance in polyploid species has an additive genetic effect [15]. In the allohexaploid bread wheat, mutations in two copies of the *AHAS* gene allow for an increase in the level of herbicide resistance compared with genotypes having only one mutated gene [22]. This work shows the first molecular characterization of the *AHAS* genes in quinoa as well as the interaction between its homeologous genomes.
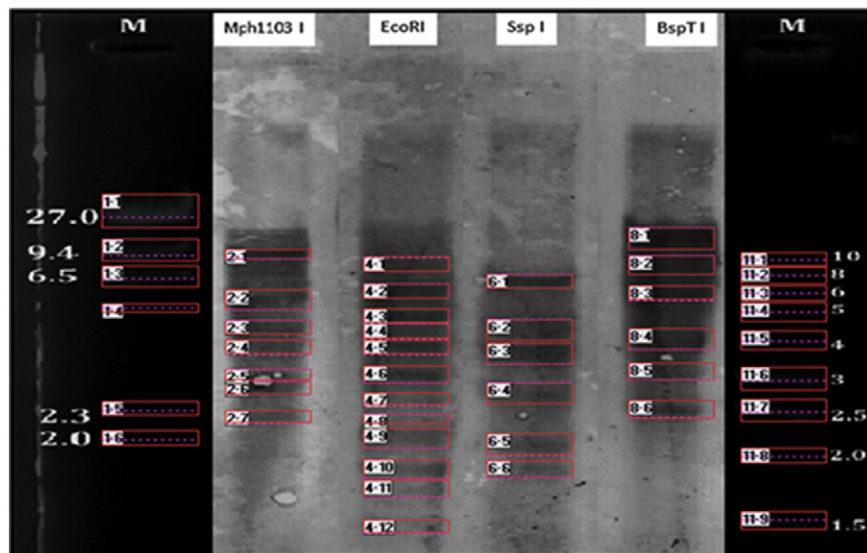
Fig. 3. Southern blot analyses using endonucleases Mph1103I, EcoRI, SspI and BspTI, six copies of the *AHAS* gene of *Chenopodium quinoa* Regalona-Baer variety were detected. M represents a molecular marker in kilo bases. The digitalized membrane indicates the number of detected bands using UN-SCAN-IT gel 6.1 software.

Fig. 4. Partial nucleotide sequences of *CqAHAS1*, *CqAHAS2* and *CqAHAS3*. Polymorphic sites are colored and similar colors show homology among sequences.

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