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Genetic variation of the granule-bound starch synthase I (*GBSSI*) genes in *waxy* and non-*waxy* accessions of *Chenopodium berlandieri* ssp. *nuttalliae* from Central Mexico

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Abstract

Huauzontle (*Chenopodium berlandieri* ssp. *nuttalliae*) is a locally important vegetable crop native to the highland valleys of Central Mexico and a potential source of genes for improving its Andean sister crop, quinoa (*Chenopodium quinoa*). A previous work involving two huauzontle lines identified one *waxy* genotype that lacked amylose due to mutations in granule-bound starch synthase I (*GBSSI*), major amylose-synthesis genes with two constituent subgenomes, A and B. We conducted this study to determine the extent of *waxy* genotypes and cryptic *GBSSI* mutations in 11 huauzontle accessions or landrace populations extending from Puebla in the southeast to Jalisco in the northwest. This represents one of the first published studies of genetic variation in *C. berlandieri* ssp. *nuttalliae*. Accessions were phenotyped for opaque versus translucent seed morphology and their seed starches were stained with Lugol's Stain. In addition, complete or partial *GBSSI* genes from their A and B genomes were polymerase chain reaction (PCR)-amplified, cloned and sequenced. Seven accessions were either wholly or partially non-*waxy* while six were either entirely or partially *waxy*. All *waxy* accessions carried the same putatively null alleles, designated *gbssIa-tp* (A-genome) and *gbssIb-del* (B-genome). The identification of publicly available genotypes carrying *gbssIa-tp* and their potential use in breeding *waxy* grain quinoa is discussed.

Keywords: *Chenopodium berlandieri* ssp. *nuttalliae*; *GBSSI*; huauzontle; quinoa; starch; *waxy*

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Introduction

The goosefoot genus *Chenopodium* (Chenopodiaceae, $x = 9$) has a worldwide distribution (Bhargava *et al.*, 2006). The foliage constitutes a rich source of carotenoids, minerals and vitamin C (Prakash *et al.*, 1993; Bhargava *et al.*, 2010). Quinoa (*Chenopodium quinoa*) seeds have protein in the 12.8–15.7% range, with elevated amounts of the essential amino acids threonine, lysine, methionine and tryptophan (Repo-Carrasco *et al.*, 2003).

Chenopodium berlandieri is a goosefoot that was domesticated at least three times anciently in Eastern North America: (1) as a thin-testa form of *C. berlandieri* ssp. *jonesianum*; (2) a thick-testa form of *C. berlandieri* that may have been utilized as a leafy vegetable and (3) another thin-testa seed crop morphologically similar to a modern Mexican domesticated chenopod, *C. berlandieri* ssp. *nuttalliae* cv. 'huauzontle' (Smith and Yarnell, 2009; Jellen *et al.*, 2011). Genetic data from hybridization studies (Wilson, 1990), karyotype analyses (Bhargava *et al.*, 2006; Palomino *et al.*, 2008; Kolano *et al.*, 2011) and gene sequencing (Maughan *et al.*, 2006; Storchova *et al.*, 2014; Walsh *et al.*, 2015) indicate that *C. berlandieri*, its South American weedy ecotype *Chenopodium hircinum*, and Andean *C. quinoa* form a New World biological species complex of allotetraploids ($2n = 4x = 36$), whose two subgenomes, designated A and B, likely originated from diploids in the New World and Old World, respectively. In addition to vegetable huauzontle, subspecies *nuttalliae* also includes seed and semi-weedy cultigens in Mexico (García-Andrade and De La Cruz, 2011).

Starch, a vital energy component of seeds, consists of molecules of α-D-glucopyranose in two different types of polymers: linear, helical α-1,4 linked chains of insoluble amylose; and mixed α-1,4 and α-1,6 branched, water-soluble amylopectin. Atwell *et al.* (1983) and Lindeboom *et al.* (2005) reported that quinoa starch has amylose contents ranging from 3 to 20%. Amylose is synthesized by *GBSS*, while amylopectin is synthesized by soluble starch synthases, starch branching enzymes and starch debranching enzymes (Park *et al.*, 2012c), in concert with *GBSSI* (Denyer *et al.*, 2001).

The *waxy* (*wx*), amylose-free seed phenotype is due to loss of function of the *GBSSI* gene. *Waxy* mutants occur in many cereals including wheat (Huang and Brule-Babel, 2012), rice (Hirano *et al.*, 1998; Crofts *et al.*, 2012) and cassava (*Manihot esculenta*; Aiemnaka *et al.*, 2012). Starches that are *waxy* or are low in amylose are desirable in rice (Liu *et al.*, 2009). Upon cooking, *waxy* starches produce a soft paste with a sticky texture, whereas wild-type starches produce a harder gel that separates easily from the cooking water (Hunt *et al.*, 2013) and can recrystallize (Denyer *et al.*, 2001).

The objective of this study was to survey the distribution of *waxy* mutants in landrace populations of huauzontle. Preliminary screening of five *C. quinoa* varieties failed to identify any with a *waxy* phenotype; however, one out of two huauzontle populations, designated 'H-02', was *waxy* (Brown *et al.*, 2014). We hypothesize that huauzontle, being a vegetable crop, was not subjected to as stringent a level of selection pressure for seed quality as quinoa.

Materials and methods

Plant materials and starch phenotyping

In this study, we worked with 11 Mexican populations or distinct cultigens of *Chenopodium berlandieri* ssp. *nuttalliae*: (1) H3, Atlacomulco, Mexico State, 19°48′N, 99°52′W, 2570 masl; (2) H5, Tenango del Valle, Mexico State, 18°39′7″N, 99°31′37″W, 2600 masl; (3) H7 opaque, El Capulín, Otzolotepec, Mexico State, 19°25′55″N, 99°33′28″W, 2580 masl; (4) H9, Zolotepec, Xonacatlán, Mexico State, 19°24′N, 99°32′W, 2610 masl; (5) H17 translucent, La Concepción Huchochitlán, Toluca, Mexico State, 19°37′32″N, 99°39′14″W, 2680 masl; (6) H18 translucent, La Concepción Huchochitlán, Toluca, Mexico State, 19°37′32″N, 99°39′14″W, 2689 masl; (7) H35-08 translucent, San Andrés Cuexcontitlán, Mexico State, 19°22′08″N, 99°36′40″W, 2670 masl; (8) PI 433230, Guadalajara, Jalisco, 20°37′46″N, 103°22′24″W, 1500 masl; (9) PI 433231, Atlixco, Puebla, 18°53′45″N, 98°21′41″W, 1880 masl; (10) PI 568155 Cacaloxuchil, Puebla, 18°45′0″N, 98°30′0″W, 1680 masl and (11) PI 568156, Acutzilapan, Mexico State, 19°47′0″N, 99°41′0″W, 2700 masl (Fig. S1, available online). Accessions H17 and H18 were collected together in the same field, but they are distinct cultigens.

The approximate content of both amylose and amylopectin was analysed by staining with Lugol's I_2–KI Stain (0.1 g resublimated iodine and 0.2 g KI dissolved in 30 ml distilled water) following the protocol developed by Brown *et al.* (2014), who had previously demonstrated the utility of this technique in *Chenopodium*. Lugol's Stain is a common tool for discriminating between non-*waxy* (purple-blue) and *waxy* (red-brown) seeds, for example, in the grain amaranths (Park *et al.*, 2012a, b). Inspection of stained quinoa starch suspensions from powdered seed was performed under 630–1000 × magnification using an Axioskop 2 microscope (Zeiss, Jena, Germany).

Populations one through seven of *C. berlandieri* ssp. *nuttalliae* listed above were obtained from the Instituto Nacional de Investigaciones Nucleares (ININ) in Ocoyoacac, Mexico. Most of these accessions contained seeds of a mixture of phenotypes, including seeds that were translucent, opaque and of various colours (mostly brown, orange-red or black). Since previous studies by Park *et al.*

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(2012a, b) had noted an association between opaque/non-*waxy* and translucent/*waxy* perisperm, respectively, in grain amaranths (*Amaranthus* spp.) – which are in the same family, *Amaranthaceae*, as *Chenopodium* – we wanted to see whether this association also held true in huauzontle. Translucent seeds were selected, with the aid of a transmitted light box, for accessions H3, H5, H9, H17, H18 and H35-08. Opaque seeds were selected from accession H7. Population H9 contained black and yellow-translucent seeds and the staining with iodine solution was different between them; for that reason, we sequenced *GBSSI* from DNA of plants derived from both seed phenotypes in this population, yellow-translucent and black. Hence, after raising single plants from selected seeds of known huauzontle phenotypes, we obtained the whole sequence of *GBSSI* representing eight different seed phenotypes from seven accessions. Considering that all of the foregoing populations of *C. berlandieri* ssp. *nuttalliae* are property of the Mexican government and are, therefore, restricted germplasm sources, we decided to also starch-phenotype and sequence the A-genome homoeoallele in four strains of huauzontle from the United States Department of Agriculture-National Plant Germplasm System (USDA-NPGS) collection to identify potential lines that could be used as parents in developing publicly available breeding lines. This group included: PI 433230, PI 433231, PI 568155 and PI 568156 (Fig. S1, available online). The 512 bp portion of *GBSSI* from the start codon to the end of exon 2 was sequenced in these accessions.

DNA extraction

Seeds of each population were germinated in the Life Sciences Greenhouse at Brigham Young University, Provo, UT, and young leaves were collected. Fresh leaves of three plants were lyophilized, individually, in a freeze-dryer at 0.7 atm for 2 d. Between 20 and 30 mg of dry leaves were crushed (FastPrep FP120; Bio 101 Thermo-Fisher Scientific, Waltham, MA, USA), and the DNA was extracted according to Dellaporta and Hicks (1983) and modified from Dellaporta (1993). The total concentration of DNA of each sample was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). We used only one DNA extraction of one individual from each population for polymerase chain reaction (PCR) amplification, cloning and sequencing. We selected the sample having the best DNA quality.

GBSSI *amplification and cloning*

Primers for *GBSSI* cloning were designed from amaranth and *C. quinoa* sequences (Geneious v. 6.1.6; Biomatters Ltd., Auckland, New Zealand, available from http://www.geneious.com) as described by Brown *et al.* (2014). Four segments were cloned between nucleotides 176 and 2478: first pair: 180 F, 5′-ACG CGA AAA ATC CTA CTG AGG AGC-3′ and 864 R, 5′- CAC GCT AAA TCG AAG CTG GT-3′; second pair: 646 F, 5′-TTC CAC ACC TAC AAG CGA GG-3′ and 1718 R, 5′-CAG GCA AAT GAA GAC GCG AG-3′; third pair: 1454 F, 5′-GGC ATA GTG CTC TTC TCC CAG CC-3′ and 2223 R, 5′-ACC AAC TTC TGC TTG TAG GGC TTC C-3′; fourth pair: 2020 F, 5′-ATG GAT GTC CTG GAA TGG AA-3′ and 2840 RA, 5′-CCC ATA TGG AAT CCG GTG TA-3′. The genomic DNA (100 ng per reaction) was amplified using each pair of primers in order to obtain the amplification of the *GBSSI* gene. We used One Taq 2x Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA). The PCR product was purified using a PCR clean-up system (Wizard® SV Gel and PCR Clean-Up System; Promega, Madison, WI, USA).

Subsequently, the PCR product, previously purified and quantified, was introduced into a vector system (pGEM®-T and pGEM®-T Easy Vector Systems; Promega, Madison, WI, USA). Cloning was necessary in order to separate individual sequence variants from heterogeneous PCR amplicons due to the presence of multiple genomes in the allotetraploid. Six or more selected clones were randomly chosen for sequencing per amplified fragment and for each individual (one individual per sample). We used between 50 and 100 ng/ml of DNA per 10 ml reaction volume. The ligation and transformation protocols had been described previously by Brown *et al.* (2014).

In order to amplify and sequence the ends of *GBSSI,* we employed a rapid amplification of complementary DNA (cDNA) ends (RACE) strategy (Yeku and Frohman, 2011) using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA), following the manufacturer's protocol. The mRNA for this procedure was extracted from immature inflorescences (green and pre-anthesis) collected from *C. quinoa* and *C. berlandieri* plants as described in Brown *et al.* (2014). The inflorescences were immediately frozen in liquid nitrogen and subsequently stored at −80°C until use. A Qiagen kit was used for RNA extraction (RNeasy, Plant Mini Kit 50 Cat. No. 74904, CA, USA). At the end, RNA was quantified in a Nano Drop Spectrophotometer (ND-1000, V3. 8.1, 2010, Thermo Scientific, Wilmington, DE, USA).

Transferrin receptor (TFR) primers were designed based on the *C. quinoa* sequence; we designed different primers for different places at the start and end of the two homoeologous genes. Primers for the 5′-end of the genome A copy were 20F/736R and 20F/2171R. Primers for the 3′-end of the genome A copy were 1820F/3295R, 1783F/3278R and 2224F/3278R. Primers for the 5′-end of the genome B copy were -80F/1539R, 180F/

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1537R and -80F/624R. Primers for the 3′-end of the genome B copy were 1200F/3415R, 1300F/3276R and 1300F/3334R. The PCR amplicons obtained with these primers were cleaned by the phosphatase/exonuclease protocol (New England Biolabs, Ipswich, MA, USA).

DNA sequence analysis

Sequences generated by Sanger sequencing as described by Brown *et al.* (2014) were aligned and analysed using the Geneious software (Geneious v. 7.0.6; Biomatters Ltd., Auckland, New Zealand, available from http://www.geneious.com). The first step was identification of A and B subgenomes in order to align sequences by subgenome groups (Brown *et al.*, 2014). Previously, both A- and B-genome variants were obtained by sequencing diploid species of *Chenopodium*. Genome A was sequenced from the North American diploid *Chenopodium standleyanum* and Genome B (BB) from Eurasian *Chenopodium ficifolium*. We measured the pairwise % identity of each huauzontle *GBSSI* sequence and the higher of the two identity values when compared with the two diploids was used to match that sequence with either the A or B genome – usually, between 97.5 and 100% for the A genome and approximately 97.5% for the B genome, as had been previously performed with *C. quinoa* and *C. berlandieri* by Brown *et al.* (2014).

We sequenced between three and six amplicon-containing colonies per population to capture both the A and B subgenomes. After their classification in subgenome A or B, we aligned all the segments, and total *GBSSI* sequence was obtained for each of the accessions or cultigens. After sequencing flanking ends of the genes with the RACE reaction protocol (Brown *et al.*, 2014), the complete coding sequence could be identified. Sequence annotation was performed (i.e. identification of intron/exon junctions, and start and stop codons) by comparison with sequences of *C. quinoa* and *C. berlandieri* previously reported by Brown *et al.* (2014) and by comparison with *Amaranthus* sequences deposited in NCBI (http://www.ncbi.nlm.nih.gov).

Results

Starch staining

Potassium iodide staining of each of the eight cultigens showed clear differences in the staining among them (Fig. S2, available online). The presence of stained granules in brown, purple or blue was observed in each of the eight varieties or cultigens, but in different proportions. The percentage of both purple and blue granules was 83% for H3 translucent, 75% for H7 opaque, 87% for H9 black, and 80% for the H18 translucent red seed. Three cultigens showed a high percentage of starch granules stained brown. The proportion of granules stained brown was 98% for H5 translucent, 87% in H9 translucent and 88% in H17 translucent. Only one of the cultigens showed an intermediate staining pattern. Sample H35-08 stained 50% purple-brown and 50% brown. According to these results, we identified the following as non-*waxy* cultigens: translucent H3, opaque H7, black H9 and translucent H18. We identified cultigens translucent H5, translucent H9 and translucent H17 as being *waxy*. The H35-08 cultigen was classified as intermediate – perhaps as low-amylose – cultigens (Fig. S2, available online). Subsequently, PI's 433231 and 568155 were observed to be staining brown (*waxy*), while 433230 and 568156 stained purple or non-*waxy* (results not shown). Seed starch phenotypes are indicated by accession label colour on the map in Fig. S1 (available online).

GBSSI *in* Chenopodium berlandieri *var.* nuttalliae

After sequencing the ends of the gene *GBSSI* with RACE methodology, we were able to identify both the start and stop codons (Brown *et al.*, 2014). The prediction of exon/intron sites was facilitated by the relatedness of the genera *Chenopodium* and *Amaranthus*, both of them being confamilial in the family *Amaranthaceae*. The identity of the *GBSSI* gene of *C. berlandieri* (A or B genome) in comparison with the *Amaranthus hypochondriacus* genome was 80–84%. The identity was essentially the same when *C. berlandieri* was compared with close relatives of *A. hypochondriacus, Amaranthus cruentus* or *Amaranthus caudatus*. The alignment of the coding regions of *A. caudatus*, *A. hypochondriacus* and the eight accessions or cultigens of *C. berlandieri* var. *nuttalliae* with their entire *GBSSIa* and *GBSSIb* genes sequenced revealed a pairwise % identity of 92.5, with 79.2 % of sites identical (481 sites) for *GBSSIa*. The B genome of *C. berlandieri* was very different, having a pairwise % identity of 85.3, with 67.9% of sites being identical (412 sites) (Fig. S3, available online). In huauzontle, the A genome of *GBSSI* had a total of 13 exons – exactly the same as in *C. quinoa* (Brown *et al.*, 2014). These exons were present in all eight of the cultigens, the number of nucleotides being constant in each one (Table 1). The sum of the nucleotides of the 13 exons within the A genome was 1818 (including the stop codon), which resulted in a coding sequence of 605 amino acids (minus Ter). The eight cultigens showed only slight amino acid sequence differences between non-*waxy* and *waxy* accessions (Fig. 1).

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Table 1. Combinations of sequence variants in huauzontle and their associations with seed morphology and starch phenotype

			A-genome variants				B-genome variants	
Cultigen and seed morphology	Starch phenotype	Genome designation	54	274	325	456	Deletion	417
H3 T	Non-*waxy*	A	Ile/Thr	Pro	Ile/Val	Leu/Val	–	–
		B	–	–	–	–	Del	Glu
H5 T	*Waxy*	A	Thr	Ser	Val	Leu	–	–
		B	–	–	–	–	Del	Glu
H7 O	Non-*waxy*	A	Thr	Ser	Val	Leu	–	–
		B	–	–	–	–	Wt	Ala
H9 T	*Waxy*	A	Thr	Ser	Val	Leu	–	–
		B	–	–	–	–	Del	Glu/Ala
H9 B	Non-*waxy*	A	Thr	Ser	Val	Leu	–	–
		B	–	–	–	–	Wt	Ala
H17 T	*Waxy*	A	Ile	Pro	Ile	Val	–	–
		B	–	–	–	–	Del	Glu
H18 T	Non-*waxy*	A	Thr	Ser	Val	Leu	–	–
		B	–	–	–	–	Wt	Ala
H35-08 T	Non-*waxy*	A	Thr	Ser	Val	Leu	–	–
		B	–	–	–	–	Del	Glu

Glu, glutamic acid; Ala, alanine.
Seed morphology: T, translucent; O, opaque; B, black.
Other explanations: Del, deletion present; Wt, wild-type (no deletion).

The *waxy* genotypes H3 and H17 shared three similarities: a Pro (vs. Ser) residue at position 274, an Ile (vs. Val) residue at position 325 and a Val (vs. Leu) residue at position 456. Additionally, within landrace accession H3, there were three polymorphisms, indicative of heterozygosity within the sampled plant: the first was ambiguity for Thr or Ile at position 54, the second was the presence of either Ile or Val at position 325 and the third was a Leu/Val ambiguity at amino acid position 456 (Fig. 3). Since Brown *et al*. (2014) hypothesized that the Ile → Thr substitution is associated with improper plastid targeting of *GBSSIa*, we sequenced this portion of the gene in the four USDA-NPGS accessions and noted that PI 433230 was heterozygous T/C (Ile/Thr), PI 433231 was homozygous C/C (Thr), PI 558155 was homozygous C/C (Thr) and PI 558156 was homozygous T/T (Ile) at position 54.

In contrast, the B genome had a greater range of sequence polymorphisms when compared with the A genome (Figs 1–3). The alignment in Fig. 2 shows 12 sites along the sequence in which there were differences among the accessions. All of the non-*waxy* plants possessed the normal-length allele with 13 exons. However, the B genome of the *waxy* accessions had a deletion 79 amino acids in length, resulting in a hypothetical polypeptide consisting of only 11 exons. Differences between the A and B subgenome sequences for *GBSSI* are presented schematically in Fig. 3. We detected five *waxy* populations and three non-*waxy* populations in samples provided from ININ, México. The missing region was towards the 5′-end of the gene and affected the first three exons. Exon 1 was lacking the last 45 amino acids, Exon 2 was completely eliminated and Exon 3 was missing the first 7 amino acids (Fig. 2). The sum of nucleotides in the 13 exons of the B-genome allele in the non-*waxy* varieties was 1818 (including the last three nucleotides of the stop codon), for a total of 605 amino acids – the same as the A-genome alleles. However, *waxy* varieties had only 1581 nucleotides (including the last three nucleotides of the stop codon), for a total of 526 amino acids. This represents a reduction of 13% in the length of the coding region. In this way, mutations in the B genome allowed us to clearly identify *waxy* (null) and non-*waxy* gene variants. The accessions H7 opaque, H9 black and translucent H18, which we classified as non-*waxy*, showed the wild-type, 13-exon allele. The deletion mutation was detected in all landraces classified by potassium iodide staining as *waxy*: translucent H5, translucent H9, translucent H17 and even in the low-amylose translucent H35-08. Interestingly, though we had classified H3 as non-*waxy* according to potassium iodide staining, it was also homozygous for the *gbssIb-del* mutation, which suggests that presence of only one functional copy of *GBSSIa* is sufficient to confer the wild-type phenotype with respect to seed amylose.

We separately analysed two seed phenotypes in accession H9: yellow-translucent seeds that had 87% brown-staining starch granules and black seeds having 87% purple-blue starch granules. In keeping with our expectations, yellow-seeded plants having the *waxy*

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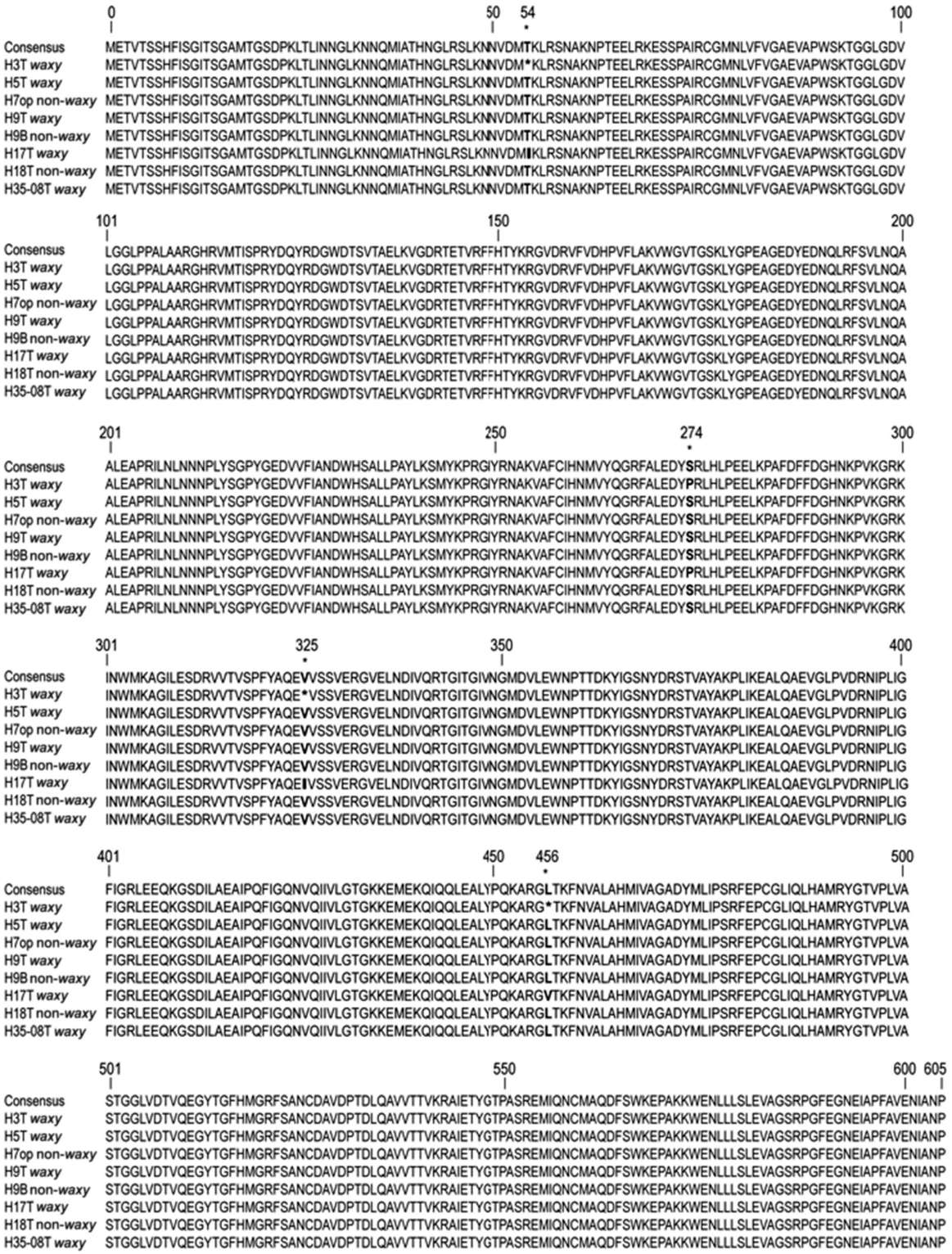

Fig. 1. Amino acid sequence of granule-bound starch synthase I (*GBSSI*), subgenome A in huauzontle (*Chenopodium berlandieri* ssp. *nuttalliae*). The total length of the wild-type allele is 605 amino acids. Three polymorphisms were detected at different positions along the amino acid chain: position 54, threonine or isoleucine (T/I); position 325, isoleucine or valine (I/V) and position 456, leucine or valine (L/V). The mutation at position 54 lies within a conserved portion of the plastid-targeting transit peptide (Brown *et al.*, 2014).

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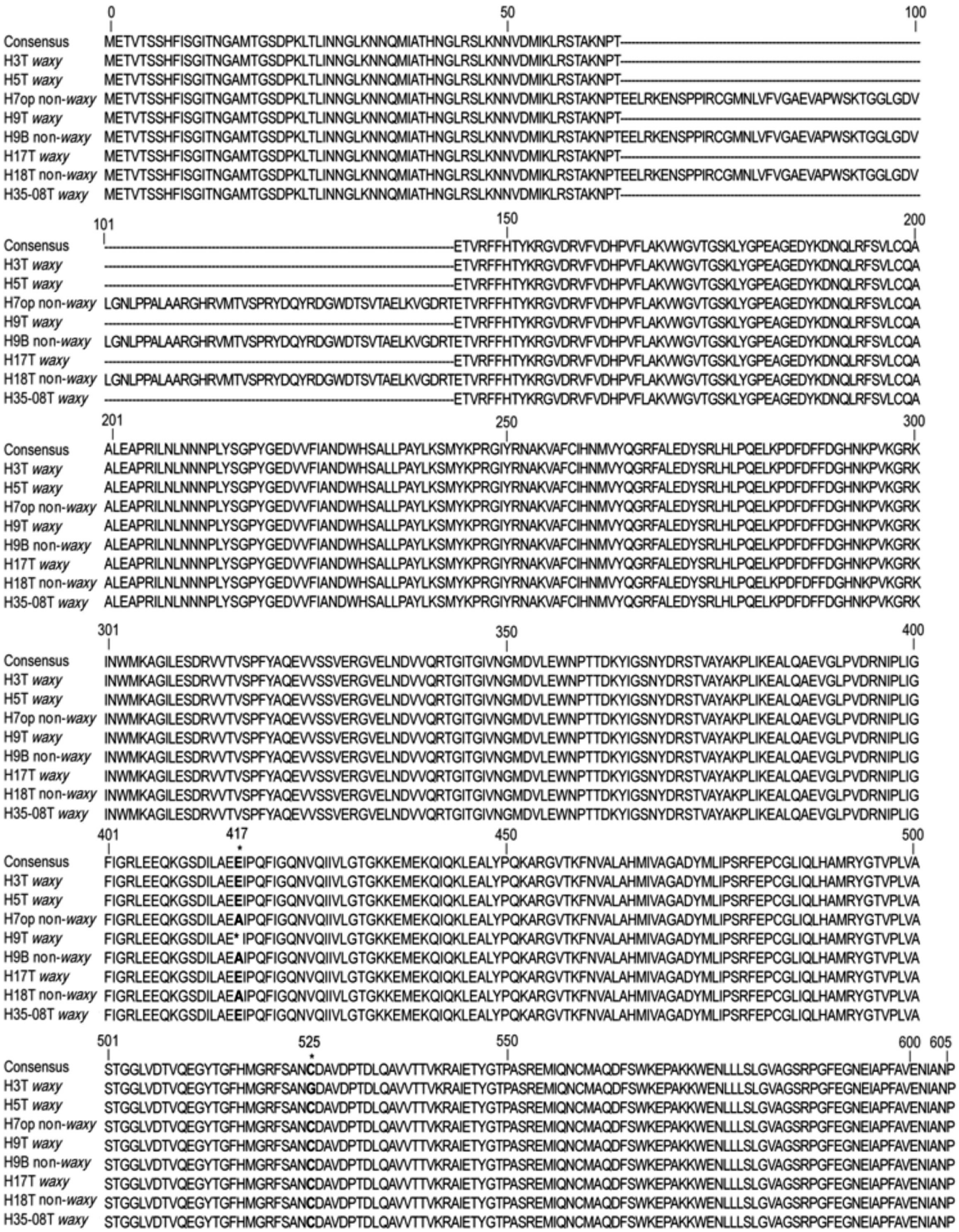

Fig. 2. Amino acid sequence of granule-bound starch synthase I (*GBSSI*), subgenome B in huauzontle (*Chenopodium berlandieri* ssp. *nuttalliae*). The total length of the wild-type allele is 605 amino acids. *Waxy* cultigens have a deletion mutation affecting amino acids 65–143. One polymorphism was also detected at position 417, where either glutamic acid or alanine (E/A) is possible.

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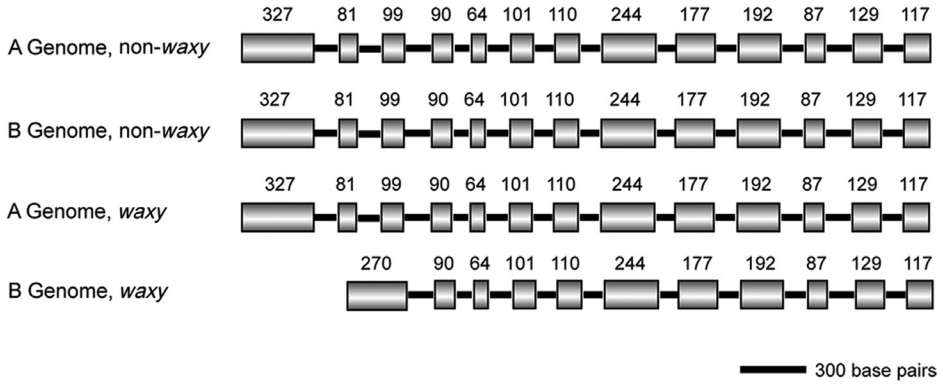

Fig. 3. Schematic of granule-bound starch synthase I (*GBSSI*) gene in base pairs of cultigens of huauzontle (*Chenopodium berlandieri* ssp. *nuttalliae*). *Waxy* genotypes are H3 translucent, H5 translucent, H9 translucent, H17 translucent and H35-08 translucent. Non-*waxy* genotypes include H7 opaque, H9 black and H18 translucent. Numbers above the boxes represent the number of base pairs in each exon of the coding region.

phenotype bore the *gbssIa-tp* (Thr at position 54) and *gbssIb-del* alleles, while black-seeded and non-*waxy* plants had the *gbssIa-tp* mutation but wild-type *GBSSIb*. However, *waxy* translucent H9 had one ambiguous result: two alternate sets of primers (−80 and −624, 180 and 1537) used to amplify the 5′-region of the gene indicated that the Ile−Thr mutation at position 54 was not present. However, it is interesting that at position 417 in the amino acid sequence of genome B, all non-*waxy* cultigens, including H9 black seed, encoded the amino acid alanine (Ala). In contrast, all of the *waxy* genotypes had glutamic acid (Glu). Interestingly, translucent H9 was heterozygous for a polymorphism at this position, having alleles encoding both Glu and Ala.

Discussion

We identified haplogroups associated with the *waxy* or low-amylose seed starch phenotypes in huauzontle (Table 1 and Fig. S2 (available online)). Individuals having the I54T-I325V-V456L haplotype in the A genome plus the large deletion in the B-genome produced less detectable (H35-08) to no (H5, H9T) seed amylose. Brown *et al.* (2014) identified the same *GBSSI* haplogroups in *waxy* huauzontle H2. Genotype H17 was anomalous, having not only all three A-genome substitutions, but also the substitution S274P. Intriguingly, this mutation, while also present in H3, had been previously reported in diploid *Chenopodium neomexicanum* accession BYU 843 by Brown *et al.* (2014). This taxon is morphologically very similar to allotetraploid *C. berlandieri* var. *sinuatum,* a wild Sonoran ecotype most similar to cultivated ssp. *nuttalliae* and therefore its candidate progenitor. The additional prior finding of Brown *et al.* (2014) that the I325V-V456L combination is in wild *C. berlandieri* ecotypes from such widespread locations as Maine (BYU 803), the Texas Gulf Coast (BYU 937), Utah (BYU 652) and northern Argentina (BYU 1101, *C. hircinum*) suggest that V325 and L456 may more accurately be considered the wild-type A-genome haplotype, albeit without the I54T substitution.

Several reports have found the existence of a transit peptide in the first 77 amino acids of the gene *GBSSI*. The *GBSSI* sequence in *Amaranthus cruentus* had 606 amino acid residues, including a transit peptide of 77 amino acids (Park *et al.*, 2009). In tartary buckwheat (*Fagopyrum tataricum*), also a pseudocereal, the genomic sequence of *FtGBSSI* contained 3947 nucleotides and was composed of 14 exons and 13 introns (Wang *et al.*, 2014). Nevertheless, the sequence of deduced FtGBSSI protein contained 605 amino acids, like *C. berlandieri*. Interestingly, they discovered a cleavage site in the FtGBSSI protein sequence towards the N-terminus with a transit sequence of 78 amino acids (8.4 kDa) and a mature protein of 527 amino acids (58.2 kDa) (Wang *et al.*, 2014). The sweet potato IbGBSSI protein also contained a signal peptide of 77 amino acids (Wang *et al.*, 1999).

In this study, consequently, we hypothesized that the first 77 or so amino acids constitute the transit peptide for CbGBSSIa. Under this scenario, the heterozygous I54T substitution in H3 is interesting because there is a polymorphism at this position, while the B-genome homoeoallele contains a large deletion suggestive of a null allele. These data support the hypothesis of Brown *et al.* (2014) that the presence of only one functional *GBSSIa* allele is sufficient to produce seed amylose. Future quantitative tests to measure seed amylose should verify whether or not there is a quantitative or additive reduction in amylose with decreasing doses of functional *GBSSI* alleles.

Appearance of the perisperm was an accurate indicator of content of amylose or amylopectin in the

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grain amaranths, with opaque seeds having the *waxy* mutation and translucent seeds the non-*waxy* genotype (Park *et al*., 2009). However, this same pattern (Fig. S2, available online) was not verified in either huauzontle or quinoa (Brown *et al*., 2014).

Although this study included a relatively small number of huauzontle samples, it is evident from Fig. S1 (available online) that the *gbssIa-tp* I54T and *gbssIb-del* mutations that result in *waxy* huauzontle are distributed across a broad geographic area and do not appear to follow a discernable pattern of grouping. Unfortunately, we know very little about the historical, let alone ancient, distribution of this crop (Wilson and Heiser, 1979). The relative commonality of the *waxy* phenotype in huauzontle is intriguing, especially since no *waxy* phenotypes were identified among 22 previously examined quinoa cultivars (Lindeboom *et al*., 2005; Brown *et al*., 2014). Lindeboom *et al*. (2005) had identified geographically diverse South American quinoa genotypes with seed amylose concentrations of 3.5–19.5%. This discrepancy can possibly be explained by one of the two hypotheses. The first possibility is that *GBSSI* mutants in one or more of the two subgenomes had already been present in a mixed weedy domesticated complex *C. berlandieri* population from which vegetable huauzontle evolved, whereas these mutations were absent in the ancestral population in South America that gave rise to quinoa. Such mutations would have no effect on phenotype because of gene duplication and the recessive nature of the *waxy* phenotype. An alternate hypothesis is that stringent selection for seed plumpness, hardiness, amylose-related cooking properties, etc., in South America eliminated *waxy* grain quinoa genotypes as they periodically emerged. In contrast, huauzontle in the central Mexican highlands was selected as a vegetable, rather than a grain, crop, hence accumulating seed quality mutations would not have been culled out as stringently as in the quinoa growing regions of South America. If *waxy* mutations reduced the cooking time of more mature huauzontle inflorescences, they might even have been unconsciously selected.

Future research will include efforts to combine the *gbssIa-tp* mutant allele with mutant B-genome alleles from quinoa to produce *waxy* quinoa cultivars. Our preliminary screen of four publicly available huauzontle accessions from the USDA-NPGS identified three accessions – PI 433230, PI 433231 and PI 558155 – that are either homogeneous or heterogeneous for *waxy* mutations and carry the *gbssIa-tp* allele. Brown *et al*. (2014) identified a single putatively null B-genome allele, designated *gbssIb-t* (W129X), in lowland quinoa genotype 'G205-95'. A PI 433231 × 'G205-95' F_2 population, for example, would be expected to harbour *waxy*:non-*waxy* plants at a ratio of 1:15 due to duplicate-dominant segregation. Known low-amylose genotypes such as 'Ames 21926' and 'Baer' will also be screened for *GBSSI* mutations (Lindeboom *et al*., 2005). Such mutations will be especially important for breeding *waxy* quinoa because of the general lack of acceptance of transgenic crops in several Latin American countries.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1479262115000076

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