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Potential Use of Wild Einkorn Wheat for Wheat Grain Quality Improvement: Evaluation and Characterization of *Glu-1*, *Wx* and *Ha* Loci

Ana B. Huertas-García, Laura Castellano, Carlos Guzmán and Juan B. Alvarez *

Departamento de Genética, Escuela Técnica Superior de Ingeniería Agronómica y de Montes, Edificio Gregor Mendel, Campus de Rabanales, Universidad de Córdoba, CeiA3, ES-14071 Córdoba, Spain; b42hugaa@uco.es (A.B.H.-G.); a02caall@uco.es (L.C.); carlos.guzman@uco.es (C.G.)
* Correspondence: jb.alvarez@uco.es

Abstract: Wild einkorn (*Triticum monococcum* L. ssp. *aegilopoides* (Link) Thell.) is a diploid wheat species from the Near East that has been classified as an ancestor of the first cultivated wheat (einkorn; *T. monococcum* L. ssp. *monococcum*). Its genome (A^m), although it is not the donor of the A genome in polyploid wheat, shows high similarity to the A^u genome. An important characteristic for wheat improvement is grain quality, which is associated with three components of the wheat grain: endosperm storage proteins (gluten properties), starch synthases (starch characteristics) and puroindolines (grain hardness). In the current study, these grain quality traits were studied in one collection of wild einkorn with the objective of evaluating its variability with respect to these three traits. The combined use of protein and DNA analyses allows detecting numerous variants for each one of the following genes: six for *Ax*, seven for *Ay*, eight for *Wx*, four for *Gsp-1*, two for *Pina* and three for *Pinb*. The high variability presence in this species suggests its potential as a source of novel alleles that could be used in modern wheat breeding.

Keywords: diploid wheat; genetic resources; puroindolines; seed storage proteins; waxy protein

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1. Introduction

In recent decades, the rise in environmental awareness has led to a new paradigm in agriculture, where the concept of sustainability is basic to all productive processes. This sustainability is intimately linked to the conservation and utilization of plant genetic resources; in fact, agriculture cannot be considered to be sustainable if it does not include a suitable program for conservation and evaluation of crop genetic resources [1]. At the same time, global warming has become one of the main challenges facing crop improvement programs [2,3]. The search for genetic materials adapted to the prevailing stressful environmental conditions without reducing the technological quality is key for the development of new wheat cultivars.

Wheat quality is a complex characteristic that depends on consumer preferences, the product and its processing; however, from a technical point of view, three grain components play an important role, with the storage proteins, the starch synthases and the puroindolines being key. Each one of these components has effects on different aspects of wheat quality. The storage proteins are mainly responsible for the dough visco-elastic properties (strength and extensibility of the gluten) [4]; the starch synthases affect the composition and properties of the starch [5]; and the puroindolines are related to the grain texture and indirectly to the capacity of the flour to absorb water due to the damaged starch granules generated during the process of milling [6]. Variation in these grain components, therefore, modulates the properties of the flour.

The degree of variation differs markedly between each of these grain components. While the storage proteins exhibit considerable genetic polymorphism, other components show more moderate levels of variation [4–10]. Of the storage proteins, the high-molecular weight glutenin subunits (HMWGs) have been the most studied due to their marked influence on the properties of the gluten [11] and the ease with which the proteins encoded by individual alleles can be distinguished. These proteins are synthesized by genes located on the long arm of the chromosome-1 group (*Glu-1* locus) [12]. The numerous studies carried out on these genes have shown that the least variation is present at the *Glu-A1* locus in cultivated hexaploid wheat [4]. Similar results have been observed for the *Wx-A1* gene, one of the three genes that code for the three waxy proteins present in bread wheat (*Wx-A1*, *Wx-B1* and *Wx-D1*). The waxy proteins are responsible for amylose synthesis in the flour starch, and their variability (between homeologs and between different alleles of the same gene) has a considerable effect on starch properties [5]. With respect to the puroindolines (puroindoline-a and puroindoline-b), these genes (*Pina* and *Pinb*) are included in the *Ha* (hardness) locus, formed by both genes together with *Gsp-1* (grain softness protein) and seven other genes without a known function [13]. In hexaploid wheat, these genes are exclusively derived from the D genome because the puroindolines genes from the A and B genomes had been deleted during the evolution event that generated tetraploid wheat [13], and therefore there is no allelic variation for these genes on the group A chromosomes.

The search for alternative gene sources is one of the strategies used to develop cultivars more adapted to perform well under the conditions of global warming. In this context, ancient wheats and wild wheat relatives, which are adapted to be grown in marginal zones under extreme conditions [14], are considered to host interesting genetic variability that could be exploited in breeding programs.

Among the potential variation sources associated with greater adaptation to adverse conditions, the wild wheat relatives carrying the A genome of polyploid wheat could be good candidates [15]. Wild diploid wheat is represented by two main species: *Triticum monococcum* L. ssp. *aegilopoides* (Link) Thell. (syn. *T. boeoticum* Boiss.) and *T. urartu* Thum. ex Gandil. Both species contain the A genome, which is closely related to the A genome of durum (*T. turgidum* L. ssp. *durum* (Desf.) Husn.) and common wheat (*T. aestivum* L. ssp. *aestivum*), although reproductive isolation between them has been indicated [16,17]. Later studies at the molecular level have suggested that, whereas *T. monococcum* spp. *aegilopoides* was the species from which cultivated einkorn (*T. monococcum* L. spp. *monococcum* L.) was domesticated, the A genome of polyploid wheats is equivalent to that of *T. urartu* [18].

The revival of the interest in the ancient wheats has increased the number of surveys carried out on cultivated einkorn, mainly with respect to the nutritional and health aspects of this ancestral crop [19–25]. As the decline in this crop began in antiquity [26–28], the variation retained from domestication until the present day could be relatively scarce. This relationship would be in agreement with the low variation detected in some genes related to technological aspects, although the wild ancestor species could contain greater variation, which could be transferred to modern wheat (see [15] for a review). In these cases, although the linkage drag of deleterious alleles linked to desirable alleles in the exotic parent, as a result of the wild nature of this source material, might be a handicap [29], the notable variation in both technological quality aspects and adaptive traits could compensate for any negative impact of the linkage drag. In this respect, the development of DNA markers that facilitate the selection of alleles of interest in a breeding program by marker-assisted selection will be key [30,31].

In the present study, we studied the variation in the loci *Glu-A^{m}1*, *Wx-A^{m}1* and *Ha* in a collection of 170 accessions of wild einkorn (*T. monococcum* ssp. *aegilopoides*) from Iran, Iraq and Turkey, with the aim of establishing the potential value of this wild species as a gene donor source for wheat quality improvement. Parallelly, the main allelic variants of each gene were characterized by diagnostic markers for evaluating this species' utility in wheat breeding.

2. Materials and Methods

2.1. Plant Material

One hundred and seventy accessions of wild einkorn wheat from Turkey, Iran and Iraq were analyzed in the current study (Table S1). These materials were kindly supplied by the National Small Grain Collection (Aberdeen, ID, USA).

2.2. Glutenin Analysis

Proteins were extracted from crushed endosperm according with the procedure described by Alvarez et al. [32]. Precipitate glutenin subunits were solubilized in buffer (ratio 1:5 mg/μL to wholemeal) and fractionated by electrophoresis in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH: 6.8/8.8) at a polyacrylamide concentration of 8% (*w*/*v*, C: 1.28%), using the Tris-HCl/glycine buffer system. Electrophoresis was performed at a constant current of 30 mA/gel at 18 °C for 45 min after the tracking dye migrated off the gel. The gels were stained overnight with 12% (*w*/*v*) trichloroacetic acid solution containing 5% (*v*/*v*) ethanol and 0.05% (*w*/*v*) Coomassie Brilliant Blue R-250. De-staining was carried out with tap water.

2.3. Waxy Protein Analysis

For waxy proteins, whole grain flour was mixed with 1 mL of distilled water and incubated at 4 °C for 24 h. The homogenate was filtered through Miracloth and centrifuged at 14,000 *g* for 1.5 min. The pellet was washed with 1 mL of buffer A (55 mM Tris-HCl pH 6.8, 2.3% (*w*/*v*) sodium dodecyl sulphate, 2% (*w*/*v*) dithiotreitol, 10% (*v*/*v*) glycerol) according to Echt and Schwartz [33]. Then, 1 mL of buffer A was added to the pellet and left for 30 min at room temperature. The pellet was washed three times with distilled water and once with acetone and then air dried. The residue was mixed with 80 μL of buffer A containing 0.02% (*w*/*v*) bromophenol blue, heated in a boiling bath for 2 min, cooled in ice and centrifuged.

Aliquots of 15 μL supernatant were loaded in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH: 6.8/8.8) at a polyacrylamide concentration of 12% (w/v, C: 0.44%). The Tris-HCl/glycine buffer system was used. Electrophoresis was performed at a constant current of 30 mA/gel and 18 °C, continuing for 4 h after the tracking dye migrated off the gel. Protein bands were visualized by silver staining.

2.4. PCR Amplification of Genes from the Glu-A1, Wx and Ha Loci

Genomic DNA was extracted from approximately 100 mg of young leaves of single plants using the CTAB (cetyl-trimethyl-ammonium bromide) method [34].

For the amplification of the genomic sequence of each gene, different strategies were used. For *Ax* and *Ay* genes, the complete coding regions of 2475 bp and 1800 bp, respectively, were amplified using the primers designed by D'Ovidio et al. [35]. The genomic sequence of the *Wx* gene contains twelve exons and eleven introns, with a coding region around 2800 bp. This genomic sequence was amplified in three fragments using the primers designed by Guzmán and Alvarez [36] and Ayala et al. [37]: the first fragment includes the 1st to 3rd exons (Wx1Fw/Wx1.3Rv); the second extends from the 3rd to the 6th exon (Wx2Fw/Rv); and the last fragment covers the region spanning the 6th to the 11th exon (Wx3Fw/Rv). These fragments overlapped between them because the Wx1.3Rv primer is the complementary sequence of the Wx2Fw primer, whereas the Wx3Fw primer is located inside the second fragment. For the *Pina* and *Gsp-1* genes, the primers designed by Massa et al. [38] were used, which generated amplicons of 516 bp and 564 bp, respectively. For the *Pinb* gene, the best results (595 bp) were obtained with the primers designed by Lillemo et al. [39].

All amplifications were performed in a 20 μL final reaction volume containing 50 ng of genomic DNA, 1.25 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM dNTPs, 4 μL 10× PCR

buffer and 0.75 U GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, WI, USA). PCR conditions as well as primer sequences are available in Table S2.

Amplification products were separated by vertical PAGE in 8% (*w*/*v*, C: 1.28%) polyacrylamide concentration gels in a discontinuous Tris-HCl buffer system (pH 6.8/8.8). The bands were stained with GelRed™ nucleic acid stain (Biotium, Fremont, Canada) and then visualized under UV light.

2.5. DNA Diagnotic Marker Analysis

The amplicons were digested with the specific endonucleases for each gene to detect internal differences between the different alleles. These endonucleases were selected according to previous studies carried out with these genes in wheat and other grasses [40–44]: *Hae*III and *Mbo*II for the *Ax* gene; *Dde*I and *Pst*I for the *Ay* gene; *Dde*I (fragments 1 and 2) and *Nco*I (fragment 3) for the *Wx* gene; *Dde*I for the *Gsp-1* gene; *Rse*I for the *Pina* gene; and *Bsr*BI for the *Pinb* gene.

Digested fragments were analyzed by polyacrylamide gel electrophoresis in a discontinuous Tris-HCl buffer system (pH: 6.8/8.8) with a 10% polyacrylamide concentration (C: 3.0%). The Tris/glycine buffer was used. The bands were stained with GelRed™ nucleic acid stain (Biotium, Fremont, Canada) and then visualized under UV light.

3. Results

Due to the nature of this study, as an initial exploration of the variability in genes related to the technological quality in wheat, this germplasm collection was analyzed, using a scaled strategy. Initially, all accessions were analyzed by SDS-PAGE for variation in the HMWGs (Table S1); subsequently, 14 representative accessions, carrying the available allelic variation for HMWGs, were screened to identify variation in the other two loci.

3.1. Variation and Characterization of the Ax and Ay Alleles for HMWGs

SDS-PAGE analysis of the HMWGs in grains from the germplasm collection showed that these materials contained both subunit types (x and y) of the proteins encoded by the *Glu-A^{m}1* locus (Figure 1). The A^{m}x subunits showed greater staining intensity than the A^{m}y subunits, which, in some cases, appeared as one major band together with several minor, more lightly stained bands, probably due to post-translational modifications, an effect which has been reported by other authors as being specific to einkorn [45,46].

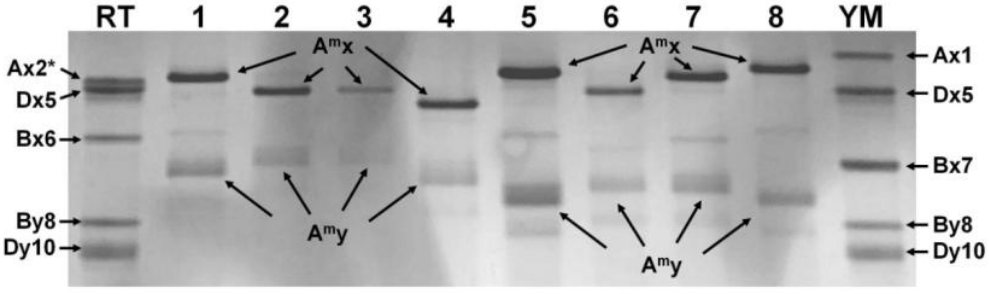

Figure 1. SDS-PAGE of representative variation for HMWGs found in the wild einkorn collection. Lanes are as follows: **RT,** common wheat cv. Rota; **1,** PI 554559; **2,** PI 427622; **3,** PI 427623; **4,** PI 554504; **5,** PI 554548; **6,** PI 427453; **7,** PI 554521; **8,** PI 470720; and **YM,** common wheat cv. Yumai-33.

In general, the A^{m}x subunits exhibited a mobility intermediate between that of the Ax1 and Ax2* subunits, although some accessions showed variants with mobility faster than either the Ax2* or the Dx5 subunit. The A^{m}y subunits have an electrophoretic mobility that is faster than the Bx subunits but lower than the by subunits (Figure 1). As the

synthesized genes of these proteins are intronless, the variation showed by DNA amplification was not different to that observed in protein SDS-PAGE separation. However, the analysis of the internal structure is easier using the nucleotide sequences rather than the extracted protein. Consequently, fourteen representative accessions containing the detected A^mx and A^my variants were evaluated by PCR amplification, followed by their digestion with specific endonucleases, which would allow the internal variation to be detected along with the relationships between these allelic variants, as previous studies have suggested [40–43]. In these accessions, both *Amx* and *Amy* amplicons were digested, showing a notable variation within the amplified sequence (Figures 2 and 3).

The *Ax* sequences were independently digested with *Hae*III and *Mbo*II endonucleases, which have been previously used to determine variation in *Ax* genes from durum and common wheat [40,41]. In this case, the variation was similar between both endonucleases, being clearer due to the size of digested fragments for *Hae*III endonuclease (Figure 2). This enzyme generates eleven fragments in Ax2* subunits and nine in Ax1 subunits, although the six larger fragments (Figure 2, lane 1) are useful to differentiate between both subunits. The main differences among both alleles are the fusion in the unique fragment of the two larger fragments at the *Ax1* allele, and the enlargement of the fourth fragment size (335 vs. 317 bp). All of these three fragments are located inside the central repetitive domain.

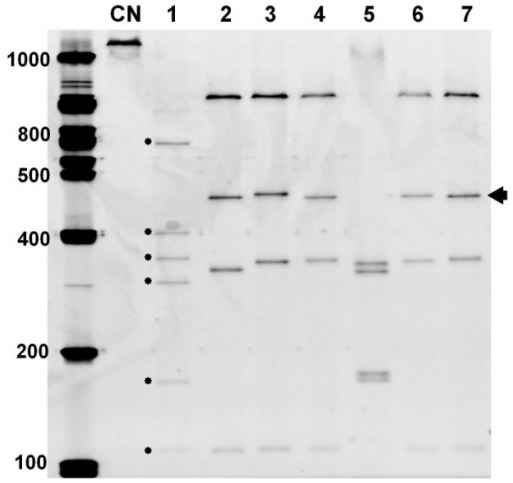

Figure 2. DNA digestion of PCR products from *Amx* alleles of the materials evaluated using *Hae*III endonuclease. The band size of the molecular weight marker is expressed in bp. Lanes are as follows: **1**, common wheat cv. Cheyenne; **2,** PI 427498; **3,** PI 427497; **4,** PI 427622; **5,** PI 554504; **6,** PI 470720; and **7,** PI 427575. **CN**, cv. Cheyenne without digestion. The digestion of the Ax2* subunit from cv. Cheyenne (●) was used as control.

The cleavage patterns of the Ax2* amplicon from cv. Cheyenne (Figure 2, lane 1) and the *Amx* amplicons detected in wild einkorn were clearly different. The *Amx* patterns were more similar to the Ax1 or Axnull amplicons, as shown by Alvarez et al. [41], although with some notable differences due to the absence of cut-off points. Thus, the fifth fragment of Ax2* that is common to Ax1 and Axnull [41] is absent in the wild einkorn alleles, with the exception of the PI 554504 accession (Figure 2, lane 5). Furthermore, these variants showed the 1279 bp fragment from the Ax1 subunit and the band equivalent to 358 bp present in all *Amx* types. However, a mutation was detected in these variants, which eliminated the cut-off point between 335 and 180 bp at the beginning of the repetitive domain (marked with an arrow in Figure 2). The use of this cleavage analysis allowed detecting up to five variants similar to Ax1 subunits (Table 1), with some differences within the

repetitive domain, together with one variant that could be more related to the Ax2* subunit (Figure 2, lane 5). The different variants detected in each accession were named with Roman numbers.

Table 1. Composition for each locus of the representative accessions evaluated.

Accession	*Ax*	*Ay*	*Wx* [F1/F2/F3] [1]	*Gsp-1*	*Pina*	*Pinb*
PI 427453	IV	IV	V [P1/P3P3]	II	I	I
PI 427497	II	VII	I [P1/P1/P1]	II	I	I
PI 427498	I	I	VII [P1/P5/P2]	II	I	III
PI 427575	VI	VI	IV [P1/P3/P2]	II	II	III
PI 427622	III	III	III [P1/P2/P1]	II	I	II
PI 427629	V	V	VIII [P1/P6/P1]	II	I	II
PI 427804	I	I	VII [P1/P5/P2]	III	I	III
PI 427963	VI	VI	VIII [P1/P6/P1]	II	I	II
PI 470713	V	V	VI [P1/P4/P1]	II	II	III
PI 470720	V	V	I [P1/P1/P1]	IV	II	II
PI 538544	I	I	II [P1/P1/P4]	II	I	III
PI 554504	IV	IV	IV [P1/P3/P2]	I	II	III
PI 554548	II	II	IV [P1/P3/P2]	III	I	II
PI 554559	III	III	I [P1/P1/P1]	I	I	I

[1] F1: fragment 1; F2: fragment 2; and F3: fragment 3 (see text for explication).

Further information on the internal structure of the *A^{m}y* genes was obtained by the digestion of amplified fragments obtained with the AyFw/Rv primers with two restriction enzymes, *Dde*I and *Pst*I (Figure 3a,b, respectively).

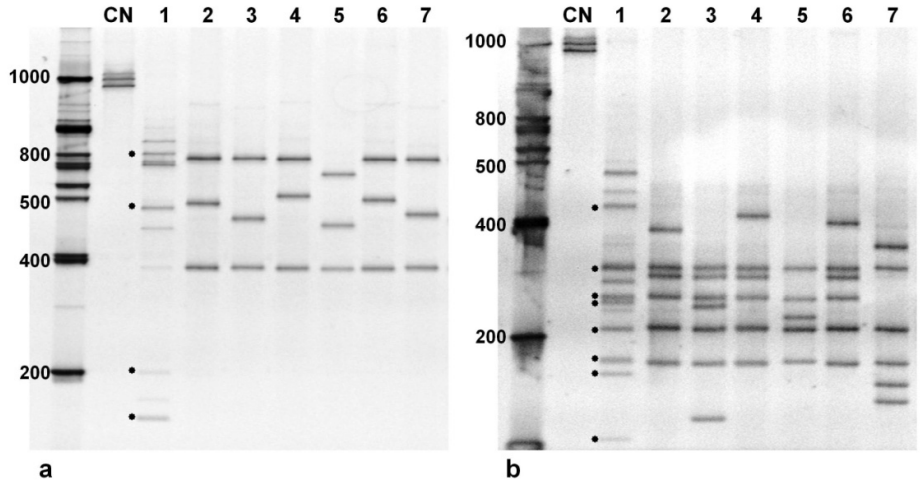

Figure 3. DNA digestion of PCR products from *A^{m}y* alleles of the materials evaluated using *Dde*I (**a**) and *Pst*I (**b**) endonucleases. The band size of the molecular weight marker is expressed in bp. Lanes are as follows: **1**, common wheat cv. Cheyenne; **2,** PI 427498; **3,** PI 427497; **4,** PI 427622; **5,** PI 554504; **6,** PI 470720; and **7,** PI 427575. **CN**, cv. Cheyenne without digestion. The digestion of the Ay subunit from cv. Cheyenne (●) was used as control.

The digestion of the ghost-*Ay* allele linked to the Ax2* subunit in cv. Cheyenne (Figure 3a, lane 1) with *Dde*I endonuclease generates up to six fragments of different sizes (in order: 5-72-164-160-564-839 bp). However, the *A^{m}y* sequences from wild einkorn present one major pattern formed by three fragments (Figure 3a, lanes 2–7), due to two point mutations, one between the 72 and 164 bp fragments and another between the 164 and 160 bp fragments [47]. These variants presented in wild einkorn did not show variation in the

size of the 72-164-160 and 839 bp fragments, with the exception of the variant detected in PI 554504 (Figure 3a, lane 5). The larger variation was detected in the central fragment (564 bp) located within the repetitive domain. The small differences in this central fragment among variants were confirmed by the use of *Pst*I endonuclease (Figure 3b). On the basis of this, the combined use of both endonucleases permitted detecting up to seven different restriction patterns associated with the same number of alleles (Table 1).

3.2. Variation and Characterization of Wx Variants

Analysis of the waxy proteins in the 12 accessions showed that these materials did not exhibit any variation in electrophoretic mobility with respect to this protein. In all cases, the accessions showed a waxy protein with an electrophoretic mobility faster than the Wx-A1 protein present in tetra- and hexaploid wheats (Figure 4).

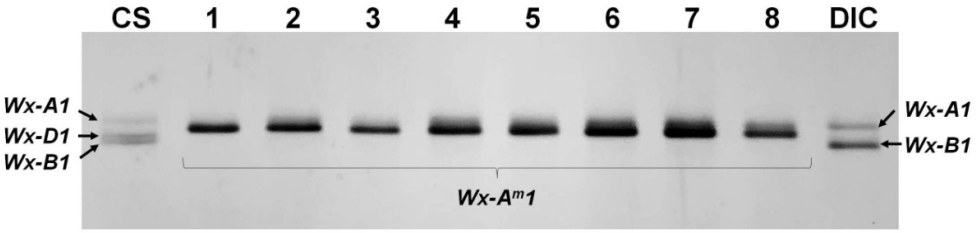

Figure 4. SDS-PAGE of representative variation for waxy proteins found in the wild einkorn collection. Lanes are as follows: **CS**, common wheat cv. Chinese Spring; **1,** PI 554559; **2,** PI 427622; **3,** PI 554504; **4,** PI 554548; **5,** PI 427453; **6,** PI 470720; **7,** PI 470713; **8,** PI 427575; and DIC, emmer wheat landrace PI 254188.

Nevertheless, although proteins' mobility was the same, the waxy genes could carry some variation at the molecular level. Due to the structure of these genes (12 exons + 11 introns), they tend to present conservative regions (mainly exons), together with variable regions (introns). As the combined use of the PCR amplification and restriction pattern of these PCR products has shown to be a useful tool to evaluate this possible variation [36,37,44], this strategy was used here (Figure 5).

The first fragment was digested with *Dde*I endonuclease that only cuts the *Wx-A1* genes, but *Wx-B1* or *Wx-D1* (Figure 5a, lanes 1 and 11). In this case, any of the variants detected in wild einkorn were digested with this restriction enzyme (Figure 5a, lanes 2–10), being consequently different to *Wx-A1* variants from durum or common wheat. This same endonuclease (*Dde*I) was used to digest the second fragment (Figure 5b). In this case, both *Wx-B1* and *Wx-D1* products were not digested (Figure 5a, lanes 1 and 11), and *Wx-A1* showed a cut-off point that generates two fragments: a very small one with 136 bp (not shown in the gel) and another with 1037 bp (Figure 5b, lanes 1 and 11). On the contrary, the *Wx-Am1* variants presented one restriction pattern very different to the reference pattern, forming two fragments with 636 bp and 550 bp (Figure 5b, lanes 2–10). Up to six different patterns were also detected: P-1, lanes 2, 6 and 8; P-2, lane 3; P-3, lanes 4–5; P-4, lane 7; P-5, lane 9; and P-6, lane 10.

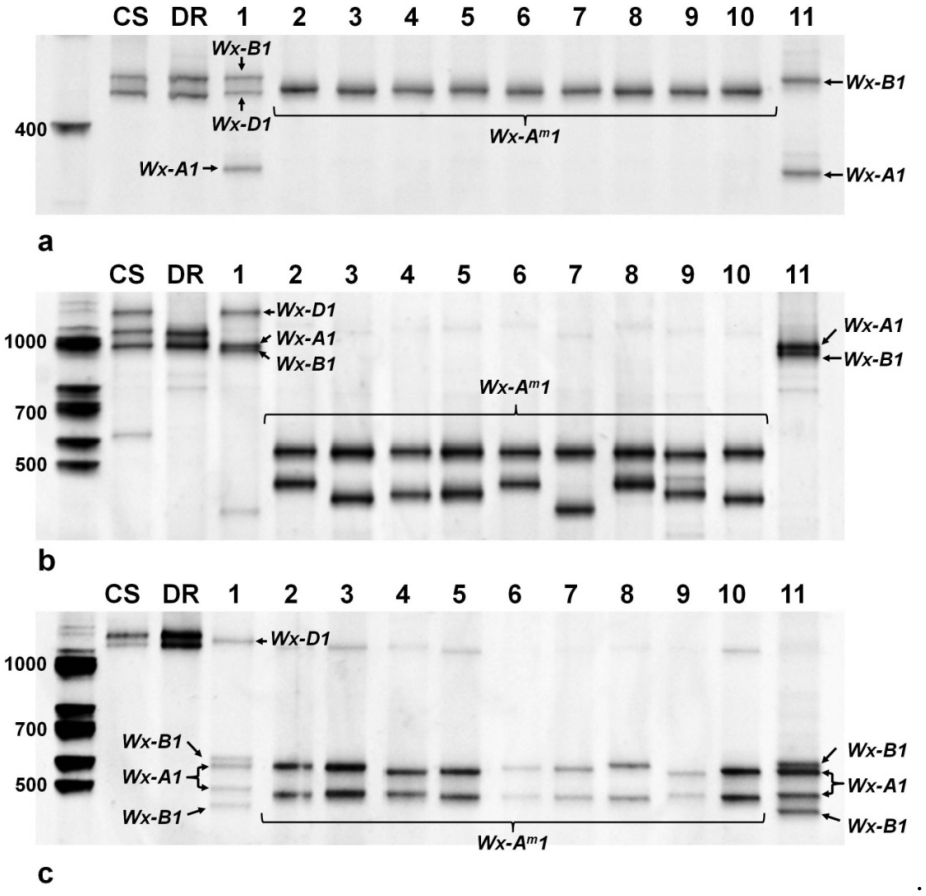

Figure 5. DNA digestion of PCR products from *Wx* alleles of the materials evaluated: (**a**) Wx1Fw/Wx1.3Rv fragment with *Dde*I; (**b**) Wx2Fw/Rv fragment with *Dde*I; and (**c**) Wx3Fw/Rv fragment with *Nco*I. Lanes are as follows: **1**, common wheat cv. Chinese Spring; **2,** PI 554559; **3,** PI 427622; **4,** PI 554548; **5,** PI 427453; **6,** PI 470720; **7,** PI 470713; **8,** PI 538544; **9,** PI 427804; **10**, PI 427629; and **11**, durum wheat cv. Don Ricardo. **CS and DR**, cv. Chinese Spring and cv. Don Ricardo without digestion.

Finally, the third fragment was digested with *Nco*I endonuclease, which did not cut the *Wx-D1* fragment (Figure 5c, lane 1), while both *Wx-A1* and *Wx-B1* were digested in two fragments that were clearly identified (Figure 5c, lanes 1 and 11). The restriction patterns of *Wx-A^{m}1* variants were similar to *Wx-A1* from durum and common wheat (Figure 5c, lanes 2–10), although showing some variation in the fragment size. This permitted detecting four different patterns: P-1, lanes 2–3, 6–7 and 10; P-2, lanes 4 and 9; P-3, lane 5; and P-4, lane 8.

The combination of three fragments with their respective digestions suggested the presence of up to eight different alleles (Table 1), although the active protein of each of them showed, as mentioned above, a similar size (Figure 4).

3.3. Variation and Characterization of Gsp-1, Pina and Pinb Variants

As a first approach, the variability of the three main genes (*Gsp-1*, *Pina* and *Pinb*) from the *Ha* locus was analyzed through amplification using the gene-specific primers designed by Massa et al. [38] and Lillemo et al. [39] (Figure 6a,b, respectively).

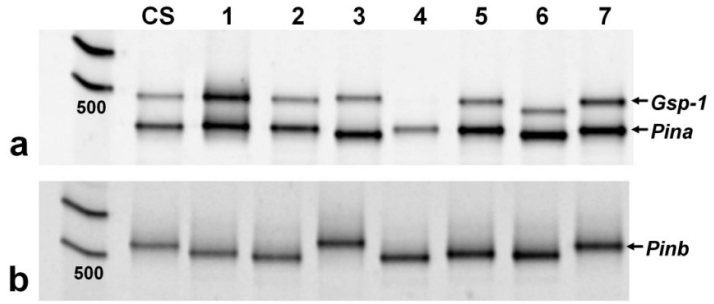

Figure 6. PCR amplification of wild einkorn lines: (**a**) *Gsp-1* and *Pina* genes, and (**b**) *Pinb* gene. Lanes are as follows: **CS**, common wheat cv. Chinese Spring; **1**, PI 554559; **2**, PI 427622; **3**, PI 554504; **4**, PI 554548; **5**, PI 427453; **6**, PI 470720; and **7**, PI 470713.

The Massa et al. [38] primers allow simultaneous amplification of the *Pina* and *Gsp-1* genes (Figure 6a), and both genes showed variation in the accessions evaluated here, presenting, in both cases, PCR products with a similar size to those observed in common wheat (Figure 6a). All accessions evaluated contained the *Pina* gene, but not the *Gsp-1* gene (Figure 6a, lane 4). According to the mobility, four variants were detected for *Gsp-1* (I: lanes 1 and 3; II: lanes 2, 5 and 7; III: lane 4; and IV: lane 6) and two for *Pina* (I: lanes 1, 2, 4, 5 and 7; II: lanes 3 and 6). For the *Pinb* gene (Figure 6b), the accessions also exhibited up to three different variants (I: lanes 1 and 5; II: lanes 2, 4 and 6; and III: lanes 3 and 7). The sum of the partial variations (*Gsp-1* + *Pina* + *Pinb*) indicated that these accessions showed marked variation for the *Ha* locus (Table 1).

A similar strategy to that used in the *Wx* gene analysis was applied here. The amplicons were digested with three endonucleases (*Dde*I for *Gsp-1*; *Rse*I for *Pina*; and *Bsr*BI for *Pinb*), which were successfully used to identify variation in these genes in previous studies [43,48].

No additional variation for *Gsp-1*/*Pina* amplicons was detected with *Rse*I and *Bsr*BI endonucleases; however, the digestion with *Dde*I was more successful (Figure 7). This endonuclease specifically cuts *Gsp-1* but not *Pina* in common wheat (Figure 7, lane CS) and in wild einkorn, with the exception of *Pina* variants present in the PI 554504 and PI 470720 accessions (Figure 7, lanes 3 and 6, respectively). Furthermore, the *Gsp-1* amplicons from wild einkorn showed one restriction pattern different to that from *Gsp-1* from cv. Chinese Spring (Figure 7, lane CS) because these amplicons did not present one cut-off point. On the other hand, the deletion of *Gsp-1* in the PI 554548 accession (Figure 7, lane 4) was also confirmed.

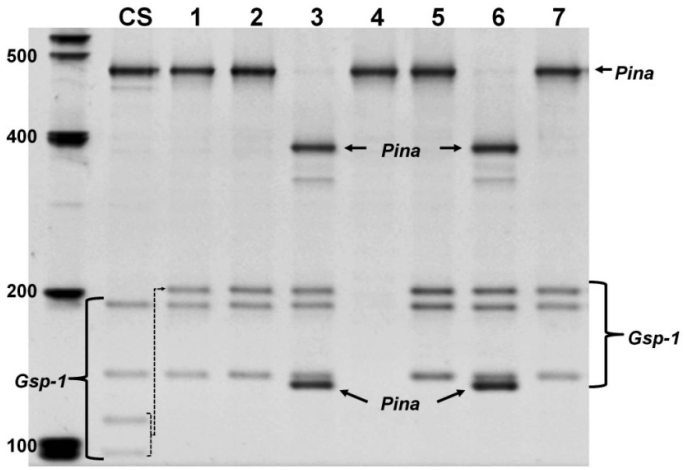

Figure 7. DNA digestion with *Dde*I of PCR products from *Gsp-1* and *Pina* gene alleles of the materials evaluated. Lanes are as follows: **CS**, common wheat cv. Chinese Spring; **1**, PI 554559; **2**, PI 427622; **3**, PI 554504; **4**, PI 554548; **5**, PI 427453; **6**, PI 470720; and **7**, PI 470713.

4. Discussion

Wild relatives of cultivated wheat have been used as sources of genes in wheat breeding programs, mainly in the improvement of biotic stress resistance [49–51]. However, the role of the primary wheat gene pool, such as the accessions studied here, in grain quality improvement has been more limited [15]. In addition to the fact that quality is a more complex character, involving many genes, than, say, disease resistance, the main difficulty is to evaluate grain quality in species that exhibit low grain yield and a very small grain. Therefore, the analysis and characterization of the genes of these wild accessions and their comparison with those of cultivated wheats may be a useful strategy, which could later lead to the introgression of these exotic genes into elite germplasms to analyze their effects on grain quality.

The differences in variation among these genes could be related to both their physiological functions in plants and to the genomic structure of each plant. The glutenin and puroindolines play roles as food reserves, structural materials or defense against pathogens in wheat grain, whereas the waxy proteins are enzymes involved in the synthesis of starch. On the other hand, intronless genes, such as those encoding glutenins or puroindolines, have a tendency to be more variable because the changes are easily fixed and translated to the mature protein. However, *Wx* genes are fragmented genes (with introns and exons), where many of the mutations occur in the introns and their effects on the properties of the protein end product are hence more limited. These differences may be useful in terms of diversity or phylogenetic studies; in fact, the *Wx* gene has been considered to be a valuable tool for this type of analysis, due to its fragmented structure and its ubiquity [52].

The variation detected for HMWGs in the wild einkorn accessions evaluated in this study was high, although some variants were present at very low frequencies and showed a clear risk of loss by random genetic drift. This variation was similar to that found in a collection of *T. urartu* accessions, which had previously been evaluated [53,54], and was clearly greater than the variation shown in some of the studies carried out with cultivated wheat species. For example, Alvarez et al. [55], using Spanish cultivated einkorn materials, detected only three *Glu-A^{m}1* variants. This low variability in the cultivated species relative to the wild ones could be related to how these materials have been used. The cultivated einkorn, although abandoned in ancient times, was cultivated for a specific purpose, probably bread making [28]. This implied a selection pressure that fixed those alleles best adapted to the use in question, while the rest of them were progressively lost, due to the fact that these genotypes were discarded, resulting in low variability [27,28]. This artificial selection process obviously did not take place in wild species (wild einkorn or *T. urartu*), and the modifications of these genes and their frequencies were regulated by only stochastic events.

Gluten strength has been positively correlated with the number of HMWGs [56,57]; in this respect, both durum and common wheat lack the alleles encoding the active Glu-1 Ay subunits and many durum wheats are also *Ax*-silent. However, wild diploid and tetraploid wheats exhibit active Ay subunits and, consequently, they could be good sources to increase the number of alleles encoding active subunits at the *Glu-A1* locus. This procedure was exploited by Rogers et al. [58] with two lines of *T. boeoticum* Boiss. ssp. *thaoudar* for introducing active Ay subunits into common wheat, in order to achieve increases in the gluten strength. This was also observed in a cross between a *T. urartu* accession and durum wheat cv. Yavaros [59]. Although further studies should be carried out, this opens the way to introgress other *Glu-A1* alleles, such as the ones found in the current study.

For the *Wx-A1* gene, our previous studies, carried out with cultivated einkorn and *T. urartu*, had shown that variation in this gene was higher in wild species than in cultivated ones. Ortega et al. [60] analyzed the genomic sequences of the *Wx-A1* genes, detecting up to five alleles in *T. urartu*, one of which had previously been described [36], but only one of which was found in the cultivated einkorn accessions analyzed, confirming the findings

of Guzmán et al. [61]. In general, the studies carried out on this gene in cultivated einkorn have shown that variation is scarce [62,63].

Grain texture is the main character controlled by the genes present at the *Ha* locus, although this locus has been mainly associated with the puroindoline genes (*Pina* and *Pinb*), whereas the role of the *Gsp-1* gene remains controversial [6,64], and the true function of the seven other genes is unknown [13]. While the diversity of puroindoline genes has been evaluated [48,65–67], there is less information available on variation in the other genes at the *Ha* locus. In the current study, the variation for both *Pin* genes was high in wild einkorn, being comparable with the variability detected in cultivated einkorn, but clearly lower than that detected in the other wild diploid wheat tested, *T. urartu* [67].

On the other hand, other notable changes have been described, such as the loss of *Pin* genes, located on the short arm of chromosome 5, in both the A and B genomes in tetra- and hexaploid wheats [13]. One clear opportunity to increase genetic variability is by re-incorporating these genes by the full or partial introgression of chromosome 5A. This process has been carried out with cultivated einkorn, the *Ha* locus of which, located on the $5A^mS$ chromosome, was transferred to cv. Chinese Spring, as either an entire chromosome [68,69] or only the *Ha* locus [70], with the grains of the resulting materials being softer than 'Chinese Spring'. This could be very interesting in durum wheat, where these materials could show a soft texture, similar to cv. Soft Svevo obtained by the 5BS–5DS translocation [71]. The incorporation of puroindoline genes from other species in durum wheat could be more effective in the A genome, given the necessity for retaining the 5BL chromosome, on which the *Ph1* gene (which controls homeolog chromosome pairing) is located, together with its lower tolerance of aneuploidy than common wheat.

The transfer of this valuable variability to the modern wheat gene pool could be achieved by different routes. This is conditioned by the homology among the A chromosomes of polyploid wheat and those of the ancestral A genome, due to the marked chromosomal reorganization which occurred after the polyploidization of wheat. One clear example of this was described by Devos et al. [72] in relation to chromosomes 4A, 5A and 7B. According to those authors, during the generation of tetraploid wheat, a reciprocal translocation occurred between chromosomes 4AL and 5AL, followed by a pericentric inversion of chromosome 4A. Finally, after a reciprocal translocation between chromosomes 4AL and 7BS, and a paracentric inversion of the original 5AL region within chromosome 4AL, the current chromosome 4AL, present in durum and common wheat, was generated. As a consequence of this reorganization, the *Wx-B1* gene, which should be on chromosome 7BS, was actually located on chromosome 4AL.

On the other hand, some differences between the genome of einkorn (A^m) and the A genome of polyploid wheats, derived from *T. urartu* (A^u), have been indicated. For this reason, crosses between einkorn (wild and cultivated) and tetraploid wheats produce offspring with A^m and A^u chromosomes. This has generated new natural species, such as *T. zhukovskyi* Menabde & Ericzjan ($A^uA^uGGA^mA^m$), an amphiploid derived from a spontaneous cross between einkorn and *T. timopheevii* (Zhuk.) Zhuk. ssp. *timopheevii* [73]. A similar occurrence was also reported by Gill et al. [74] in crosses between wild einkorn and durum wheats, which have shown potential for breeding [75]. In our previous work using *T. urartu*, several backcrosses were achieved between the hybrid and the durum wheat parent [59].

Although the data obtained in the present study should be understood as an approximation of the variability present in this wild species, our results show that the variability was high for *Glu-A1* and *Ha* loci in wild einkorn. However, for waxy proteins, these results suggest that this species is probably not the best choice of species in which to find novel variation.

5. Conclusions

Wild einkorn is an interesting source of genes related to wheat quality. Although the effects of these allelic variants on the technological quality properties in cultivated wheat should be evaluated further, the information revealed in this study may be of interest to

wheat breeders in order to select parental accessions to generate recombinant lines with different gluten and texture properties, using wild einkorn as the donor species.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/11/5/816/s1, Table S1: Origin and HMWG composition for the accessions of *T. monococcum* ssp. *aegilopoides* used in this study, Table S2: Description of PCR primer pairs for amplification.

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