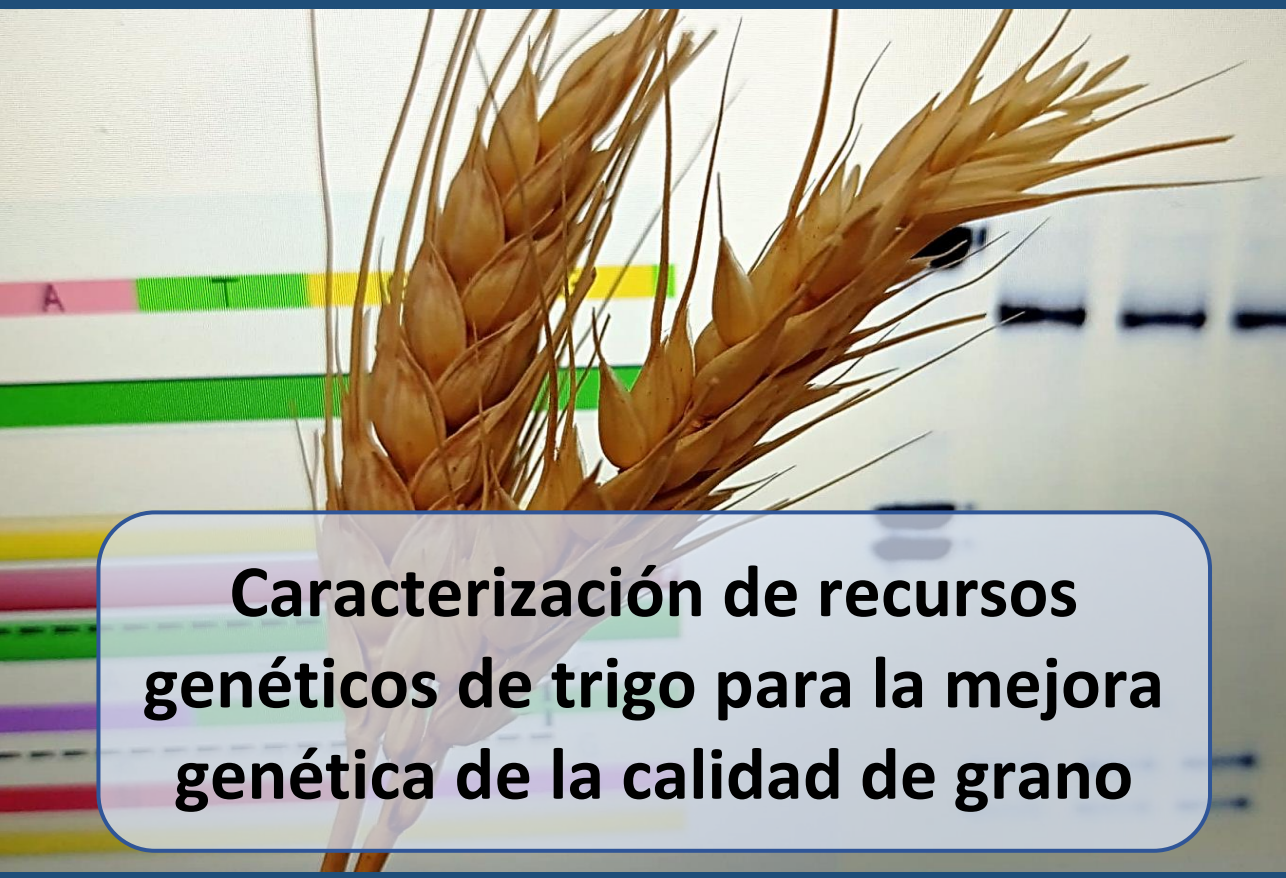
Programa de Doctorado de Biociencias y Ciencias Agroalimentarias

TESIS DOCTORAL

Ana Belén Huertas García



Caracterización de recursos genéticos de trigo para la mejora genética de la calidad de grano

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TITULO: *Caracterización de recursos genéticos de trigo para la mejora genética de la calidad de grano*

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DEPARTAMENTO

DE GENÉTICA

Programa de Doctorado de Biociencias y
Ciencias Agroalimentarias

«Caracterización de recursos genéticos de trigo para la mejora genética de la calidad de grano»

"Characterisation of wheat genetic resources for genetic improvement of grain quality"

Trabajo realizado por Ana Belén Huertas García, para optar al Título de Doctora.

Directores:
Dr. Juan Bautista Álvarez Cabello
Dr. Carlos Guzmán García

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DOCTORANDA/O

Ana Belén Huertas García

TÍTULO DE LA TESIS:

CARACTERIZACIÓN DE RECURSOS GENÉTICOS DE TRIGO PARA LA MEJORA GENÉTICA DE LA CALIDAD DE GRANO

INFORME RAZONADO DE LAS/LOS DIRECTORAS/ES DE LA TESIS
(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma)

La Tesis ha presentado un desarrollo coherente con las previsiones realizadas en su momento, con la salvedad del parón producido por las condiciones durante la pandemia que ha provocado un retraso en la culminación de la misma. La redacción y publicación de los dos últimos capítulos de la Tesis así como del documento final también se han visto algo retrasados, por contar la doctoranda con menor disponibilidad para trabajar en ello.

En esta Tesis Doctoral se ha evaluado y caracterizado la variabilidad genética para los genes y componentes implicados en la calidad del trigo en recursos genéticos procedentes de diversas regiones del mundo. Para ello se han planteado diversos objetivos: 1) Analizar la variación en los loci Glu-Am1, Wx-Am1 y Ha en einkorn de Irak, Irán y Turquía, con el fin de establecer el valor potencial de esta especie silvestre como fuente donante de genes para la mejora de la calidad del trigo. 2) Evaluar la diversidad de los genes Pina-D1 y Pinb-D1, en variedades locales de trigo harinero iraní con el fin de detectar nuevos alelos de los genes de las puroindolinas asociados a diferentes durezas del grano. 3) Analizar la variabilidad de diferentes componentes del grano con importancia nutricional en una colección española de trigo espelta y variedades modernas de trigo harinero. Y 4) Evaluar trigo espelta y variedades modernas de trigo harinero de España para características de calidad de grano relacionadas con la calidad tecnológica.

Estos objetivos han sido desarrollados a través de 4 artículos (capítulos I al IV del documento) publicados en revistas incluidas en el «Science Citation Index», bajo la modalidad de «open access».

La doctoranda ha mostrado en todo momento una gran capacidad y constancia, siendo sobre todo de destacar, el entusiasmo y la dedicación con que se ha entregado al desarrollo de esta Tesis, lo cual se ve reflejado en el número de publicaciones conseguidas. Durante el desarrollo de la Tesis, la doctoranda ha tenido que trabajar en actividades diversas (desde el manejo y cuidado de ensayos de campo, hasta trabajo de biología molecular en el laboratorio), lo que ha ido en beneficio de su formación como investigadora y lo que le permitirá afrontar futuros retos de diversa índole.

En consecuencia, estimamos que la formación de la doctoranda, así como la calidad de la Tesis están plenamente justificadas, y procede autorizar su presentación y defensa.

Lista de publicaciones.

1. Huertas-García et al. (2021). Agronomy, 11(5), 816. https://doi.org/10.3390/agronomy11050816
2. Huertas-García et al. (2022). Agriculture, 12(8), 1196. https://doi.org/10.3390/agriculture12081196
3. Huertas-García et al. (2023). Journal of Agricultural and Food Chemistry, 71(28), 10598-10606. https://doi.org/10.1021/acs.jafc.3c02365
4. Huertas-García et al. (2023). Foods, 12(16), 2996. https://doi.org/10.3390/foods12162996

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, a 5 de diciembre de 2023

Las/los directoras/es

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A mis padres y a mi abuela Victoria,

seguro los más orgullosos.

Esta tesis, mi gran sueño…

«Los sueños no se cumplen, como quien cumple años. Los sueños se madrugan, se curran, se estudian, se trabajan y un día esos sueños podrán llegar a hacerse realidad».

Toñi Acosta

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LISTADO DE PUBLICACIONES OBTENIDAS

Huertas-García, A. B., Castellano, L., Guzmán, C., & Alvarez, J. B. (2021). Potential use of wild einkorn wheat for wheat grain quality improvement: Evaluation and characterization of *Glu-1*, *Wx* and *Ha* loci. *Agronomy*, *11*(5), 816.

Huertas-García, A. B., Guzmán, C., Tabbita, F., & Alvarez, J. B. (2022). Allelic variation of puroindolines genes in Iranian common wheat landraces. *Agriculture*, *12*(8), 1196.

Huertas-García, A. B., Tabbita, F., Alvarez, J. B., Sillero, J. C., Ibba, M. I., Rakszegi, M., & Guzmán, C. (2023). Genetic variability for grain components related to nutritional quality in spelt and common wheat. *Journal of Agricultural and Food Chemistry*, *71*(28), 10598-10606.

Huertas-García, A. B., Guzmán, C., Ibba, M. I., Rakszegi, M., Sillero, J. C., & Alvarez, J. B. (2023). Processing and bread-making quality profile of Spanish spelt wheat. *Foods*, *12*(16), 2996.

Nota: Con el fin de establecer una coherencia formal a lo largo de todo el documento, se han uniformado las referencias y se han editado los trabajos originales, eliminando de ellos el apartado de referencias, el cual ha sido agrupado al final del documento.

ÍNDICE

Resumen/ Summary

Resumen

El trigo es uno de los cultivos más extendidos en el mundo. Una característica importante del mismo es la calidad del grano, que está asociada a su composición. En esta Tesis Doctoral se ha evaluado la variabilidad para diferentes genes y características de calidad del grano en diferentes recursos genéticos de trigo, de diversas especies y procedencia geográfica.

La variación para los loci *Glu-A^{m}1, Wx-A^{m}1* y *Ha* (*Pina-1* y *Pinb-1*) fue evaluada en una colección de escaña silvestre, donde se detectaron numerosas variantes para cada gen, lo que sugiere que esta especie puede ser una fuente interesante de nuevas variantes de genes relacionados con la calidad del trigo para los programas de mejora. La diversidad de los genes *Pina-D1* y *Pinb-D1* también fue evaluada en una colección de *landraces* iranís, en la que se detectaron tres alelos previamente descritos y un nuevo alelo denominado *Pinb-D1ak.*

Por último, se analizó la variabilidad de diferentes componentes del grano con importancia nutricional, calidad de procesamiento y panificación en trigos espelta y harinero. Los resultados obtenidos mostraron que existe una variación genética intra e interespecífica significativa en los compuestos asociados a la calidad de grano. En ambas especies se identificaron genotipos con valores sobresalientes para determinadas características de calidad, que podrían utilizarse como donantes en los programas de mejora para desarrollar nuevas variedades de espelta o trigo harinero con buenas características para la industria alimentaria.

Summary

Wheat is one of the most widespread crops in the world. An important trait of the wheat crop is grain quality, which is associated with its composition. In this Doctoral Thesis, variability for different genes and grain quality traits was evaluated in different wheat genetic resources from diverse geographic origin and species.

Variation was analyzed for the *Glu-A^m1*, *Wx-A^m1* and *Ha* loci in a collection of wild einkorn, in which numerous variants were identified for each gene. This suggest that this species is an interesting source of new variants of genes related to wheat quality and can be used in breeding programs as a source of new alleles.

The diversity of *Pina-D1* and *Pinb-D1* genes was also evaluated in a collection of Iranian landraces, in which three previously described alleles and the novel allele *Pinb-d1ak* were detected.

Finally, the variability of different grain components with importance on nutritional, processing and bread-making quality was analyzed in spelt and bread wheat. The results obtained showed that there is significant intra- and interspecific genetic variation in the compounds associated with grain quality. In both groups there are genotypes with outstanding values for certain quality characteristics, which could be used as donors in breeding programs to develop new spelt or bread wheat cultivars with good characteristics for the food industry.

INTRODUCCIÓN GENERAL

La producción mundial de cereales en 2023 ha alcanzado un máximo histórico, ubicándose en 2.819 millones de toneladas. El trigo es uno de los cereales más cultivados del mundo ya que representa una de las principales fuentes de alimento para el consumo humano y animal. Actualmente, el trigo, en base a su producción, es el tercer mayor cultivo a nivel mundial con 770,8 millones de toneladas (Tabla 1). Por otro lado, el trigo representa el 30,4% de la superficie total de los cereales a nivel mundial, lo que da lugar a una superficie cultivada de 220 millones de hectáreas, posicionándose así como el cereal con mayor superficie cultivada, seguido del maíz y del arroz (FAO, 2021) (Tabla 1).

Tabla 1. Datos de producción y superficie cultivada de los principales cereales.

Cultivo	**Producción (Tm)**	**%**	**Superficie (Ha)**	**%**
Maíz	1.210.235.135	39,8	205.870.016	28,3
Arroz	787.293.867	25,9	165.250.620	22,7
Trigo	770.877.072	25,3	220.759.739	30,4
Cebada	145.623.914	4,8	48.941.020	6,7
Sorgo	61.364.996	2,0	40.925.310	5,6
Mijo	30.089.625	1,0	30.934.728	4,3
Avena	22.571.618	0,7	9.562.497	1,3
Centeno	13.223.426	0,4	4.334.961	0,6

Datos según FAO (2021)

Estos datos si se comparan con años anteriores se puede considerar que la producción de trigo ha aumentado con respecto a

2015 en un 5,8% más (FAO, 2015), lo que da lugar a unos cuarenta millones de toneladas más en cinco años.

El principal país productor de trigo es China, seguido de India y de Rusia. Por otro lado, la Unión Europea también presenta unos datos de producción de trigo similar a la de los tres países previamente mencionados, en torno a 134 millones de toneladas métricas en el último año (Figura 1).

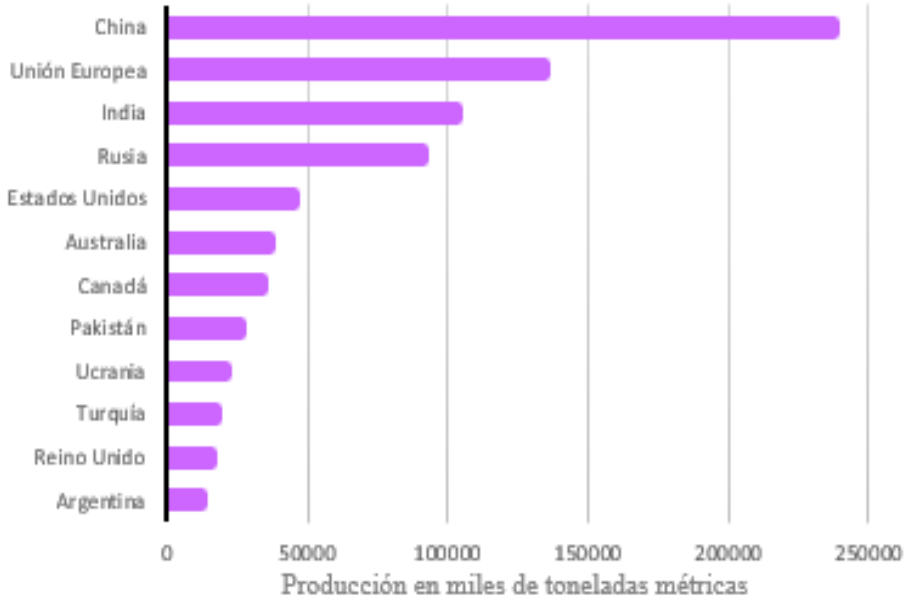


Figura 1. Datos de producción de los principales países productores de trigo.

Por tanto, con estos datos se puede concluir la gran importancia del cultivo del trigo a nivel mundial. Este éxito del trigo es debido principalmente a su gran adaptabilidad agronómica a diferentes ambientes, lo que permite su cultivo en prácticamente todos los climas y latitudes. Además, su grano es fácil de conservar y almacenar, y su harina tiene unas excelentes características que permiten la producción de una gran diversidad de alimentos (Shewry, 2009).

Origen y estructura genética del trigo

El Creciente Fértil es una región geográfica que ocupaba los territorios actuales de Israel, Palestina, Líbano, Siria, Jordania, Irak, Irán y el sudeste de Turquía (Figura 2).

Figura 2. Zona del creciente fértil, origen del trigo.

En esta zona se desarrolló la Revolución Neolítica que desencadenó la aparición de la Agricultura. Este proceso provocó una profunda transformación en las sociedades humanas que pasaron de sociedades nómadas de cazadores-recolectores a sociedades sedentarias de ganaderos-agricultores. En este contexto, las primeras plantas en ser cultivadas debido a su fácil conservación y a su elevado contenido calórico fueron los cereales y las leguminosas. Concretamente, el trigo fue uno de los primeros cereales en ser cultivado en el Creciente Fértil, ya que se remonta a hace unos 10.000 años (Feldman, 2001). Posteriormente, la Agricultura se desarrollaría en el Extremo Oriente entre el 6.500-5.500 a.C., apareciendo entre el 5.000-4.000 a.C. en América Central y la zona Andina (Harlan, 1992).

El trigo comprende un conjunto de las especies y subespecies de género *Triticum.* Dicho conjunto constituido por especies silvestres y

cultivadas se clasifica en tres niveles distintos según su nivel ploídico (Figura 3):

- Diploides: presentan un solo genoma (**A**) constituido por 7 parejas de cromosomas homólogos (2n = 2× = 14).
- Tetraploides: presentan dos genomas diferentes (**AB**), con 14 parejas de cromosomas (2n = 4× = 28).
- Hexaploides: presentan tres genomas diferentes (**ABD**) y 21 parejas de cromosomas (2n = 6× = 42).

La primera especie del género *Triticum* en ser cultivada por la Humanidad fue *Triticum monococcum* L. ssp. *monococcum* (2n = 2× = 14, $\mathbf{A^mA^m}$), especie diploide conocida como escaña o einkorn, la cual surgió por la domesticación de *T. monococcum* L. ssp. *aegilopoides* (Link) Thell. Sin embargo, ha sido otra especie diploide, *T. urartu* Tum. ex Gandil. ($\mathbf{A^uA^u}$), la propuesta como donadora del genoma **A** en las especies poliploides de trigo. Por tanto, esta especie silvestre tiene gran importancia ya que de ella procede el genoma **A** de los trigos poliploides (Dvorak *et al.*, 1993; Matsuoka, 2011).

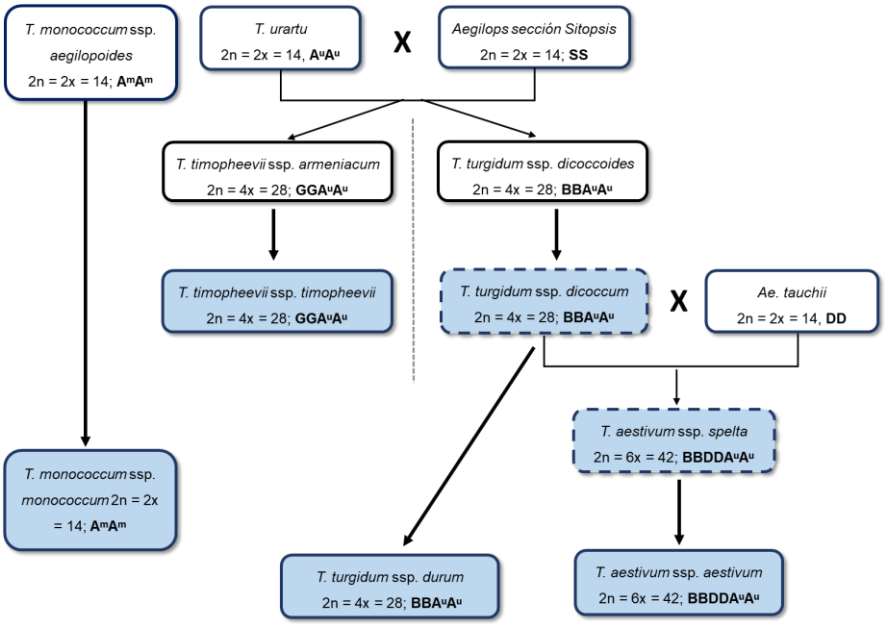


Figura 3. Origen y evolución del trigo. Las especies en azul son o han sido en algún momento cultivadas y las especies que aparecen con línea discontinua son de grano vestido.

La hipótesis más aceptada de cómo se generaron las especies tetraploides es la que sugiere que se generaron en dos eventos evolutivos distintos a partir de cruzamientos espontáneos de especies del género *Aegilops* con *T. urartu* (Dvorak and Zhang, 1990; 1992). El primer evento evolutivo, fue producido por el cruce de las especies *Aegilops speltoides* Tausch. con *T. urartu* para dar lugar a la especie tetraploide *T. timopheevii* (Zhuk.) Zhuk ssp. *armeniacum* (Jakubz.) MacKey (2n = 4× = 28, **GGA^{u}A^{u}**), cuya especie domesticada es *T. timopheevii* (Zhuk.) Zhuk ssp. *timopheevii*. Este evento ocurrió de forma local en el oeste de Georgia (Kilian *et al.*, 2007). El segundo evento evolutivo dio lugar a la aparición del emmer silvestre *T. turgidum* ssp. *dicoccoides* (Körn. ex Asch. & Graebner) Thell. (2n = 4× = 28**,**

BBA^{u}A^{u}), como consecuencia del cruzamiento de una especie perteneciente a la sección *Sitopsis* con *T. urartu.* Este hecho se piensa que ocurrió en el Creciente Fértil (Petersen *et al.,* 2006).

Posteriormente, a partir del emmer silvestre se originó el emmer cultivado *T. turgidum* ssp. *dicoccum* (Schrank ex Schübler) Thell. y a partir de éste evolucionaron el resto de los trigos tetraploides que conocemos hoy en día, entre los que destaca el trigo duro (*T. turgidum* ssp. *durum* (Desf.) Husn.). Por su parte, los trigos hexaploides se originaron por el cruce espontáneo entre emmer cultivado y *Ae. tauschii* Coss. (2n = 2× = 14, **DD),** donador del genoma **D** (Kerber and Rowland, 1974; McFadden and Sears, 1946), y la posterior selección de un agricultor, dando lugar al trigo espelta *T. aestivum* ssp. *spelta* (L.) Thell. (**A^{u}A^{u}BBDD**). Esta hibridación tuvo lugar probablemente en la zona del Mar Caspio, dentro del área de distribución natural de *Ae. tauschii* (Dvorak *et al*., 1998). Después, a partir del trigo espelta acabaría originándose el trigo común o harinero *T. aestivum* L. ssp. *aestivum*, siendo la especie más importante del género *Triticum* en la actualidad. (Figura 3)

Los usos del trigo

Desde la Antigüedad, el trigo ha sido utilizado para la elaboración de diferentes productos, pero sin duda el uso más importante ha sido la elaboración de pan. Los primeros testimonios escritos que hacen referencia al pan datan del año 2.600 a.C, aunque

se han encontrado hallazgos arqueológicos más antiguos en el noreste de Jordania hace unos 14.400 años (Arranz-Otaegui et al., 2018).

Los egipcios fueron los primeros en utilizar la levadura para la fermentación y esponjamiento de la masa en la panificación (Harlan, 1981). De hecho, en esa época el pan se usaba como un símbolo de estatus social ya que las clases altas consumían panes realizados con las mejores harinas, mientras que las clases humildes comían un pan tosco y de baja calidad (Harlan, 1981; Pomeranz, 1987). Siguiendo el proceso histórico, los conocimientos sobre panificación se fueron transmitiendo hasta llegar a los griegos y de estos a los romanos, para los cuales tuvo una gran importancia, ya que desarrollaron su producción en la cuenca del Mediterráneo a nivel industrial.

Los procesos de la panificación se realizaban de forma manual (Matz, 1960) hasta la llegada de la Revolución Industrial en la que los procesos de elaboración del pan fueron mecanizados. Este avance permitió el desarrollo de nuevas técnicas de amasado y horneado que dieron lugar a diferentes productos. Más tarde, el aumento de la población motivó a la búsqueda de nuevas variedades de trigo con mayor rendimiento y con las características óptimas para los procesos de panificación.

La forma de elaboración del pan ha continuado evolucionando hasta nuestros días, tal es así, que actualmente se dispone de una gran variabilidad de tipos de pan como pueden ser el pan sin levadura, el pan integral y miles de tipo de pan para satisfacer el gusto de todos los consumidores. Asimismo, el trigo en la actualidad tiene muchos otros

usos en la industria alimentaria ya que también es utilizado para la elaboración de pasta y couscous, a partir de sémola de trigo duro, para la elaboración de cerveza, para la elaboración de galletas y productos de repostería y, además, también se ha empezado a usar en productos no alimenticios como cosméticos e incluso para la producción de bioetanol o biomasa como cultivo energético (Bell *et al.*, 1995).

La calidad del grano

La calidad del trigo es la capacidad de una variedad de producir una harina o sémola adecuada para un producto específico. Es un parámetro variable ya que va a depender de las preferencias del consumidor, del producto que se quiera obtener (por ejemplo, las características de calidad para elaborar galletas, pasta o pan son diferentes) y de las condiciones de elaboración de un producto determinado.

A pesar de esto es un parámetro importante de definir, ya que está influenciada por las características fisicoquímicas del grano, por diferentes factores ambientales (el suelo, el clima, las técnicas de cultivo y almacenamiento) y además de un importante componente genético (Van der Veen and Palmer, 1997). Relacionado con esto, hay tres grupos de proteínas que se explicarán a continuación más en detalle dado su papel esencial en la calidad del trigo. Estas son:

- Las proteínas de reserva del endospermo (gliadinas y gluteninas)

- Las proteínas asociadas a la dureza del grano (puroindolinas)
- Las proteínas sintetizadoras del almidón (proteínas waxy)

Las proteínas de reserva del grano.

Las proteínas de reserva o prolaminas (llamadas así debido a su alto contenido en prolina y glutamina) representan el principal componente proteico del endospermo, llegando a ocupar casi el 90% de las proteínas presentes en el trigo (Shewry *et al.,* 1984).

Las proteínas de almacenamiento del grano forman el gluten. El gluten es una red viscoelástica que se forma tras hidratar y someter a agitación mecánica (amasar) la harina de trigo, y es el responsable de las propiedades viscoelásticas de la masa (Miflin *et al.,* 1983). Contiene alrededor de 80% de proteínas, 5-10% de lípidos, almidón residual, carbohidratos y proteínas insolubles en agua (Nierle and el Baya, 1990). Estas proteínas que forman el gluten pueden ser clasificadas en dos grupos principales: las gliadinas y las gluteninas.

Las gliadinas son proteínas monoméricas con un peso molecular comprendido entre 30 y 80 kDa, unidas mediante puentes de hidrógeno e interacciones hidrofóbicas. A su vez, se dividen en varios grupos en función de su movilidad electroforética en medio ácido (A-PAGE). Concretamente se dividen en cuatro grupos que son: ω-, γ-, β- y α-gliadinas según su movilidad desde el cátodo. Cada uno de estos grupos está sintetizado por genes localizados en el brazo corto de los cromosomas homeólogos 1 y 6. El loci *Gli-1* del grupo de cromosomas

homeólogos 1 (*Gli-A1, Gli-B1 y Gli-D1*) controla la mayoría de las γ- y ω-gliadinas, mientras que el loci *Gli-2* (*Gli-A2, Gli-B2 y Gli-D2*) del grupo homeólogo 6 controla todas las α/β-gliadinas (Ciaffi *et al.*, 1997; Metakovsky *et al.*, 1984; Payne et al., 1982; Wrigley *et al.*, 2006).

Las ω-gliadinas no contienen ningún residuo de cisteína, sin embargo, las α/β-gliadinas y las γ-gliadinas presentan 6 y 8 residuos de cisteína, respectivamente, que forman puentes disulfuro intramoleculares. Además, algunas mutaciones permiten a las α/β y γ-gliadinas presentar un número impar de cisteínas y poder incorporarse al gluten mediante uniones intermoleculares actuando así como terminadores de la polimerización (Barak *et al.*, 2015).

Por su parte, las gluteninas son proteínas poliméricas con un peso molecular variable, encontrándose unidas covalentemente mediante puentes de disulfuro (S-S). De acuerdo con su separación en electroforesis desnaturalizante a pH básico (SDS- PAGE) se pueden diferenciar dos tipos de subunidades: las subunidades de alto peso molecular (HMW-GS) y las subunidades de bajo peso molecular (LMW-GS) (Payne, 1987). Estas proteínas son las encargadas de otorgar a la masa elaborada a partir de la harina de trigo las propiedades viscoelásticas que caracterizan y diferencian a este tipo de harina del resto de harinas (Bonilla et al., 2020). Las HMW-GS presentan pesos moleculares entre 80-140 kDa y están codificadas por los loci *Glu-A1*, *Glu-B1* y *Glu-D1* localizados en el brazo largo de cada uno de los cromosomas del grupo homeólogo 1 (Bietz *et al.*, 1975; Lawrence and Shepherd, 1981; Payne *et al.*, 1980; Payne, 1987). Cada locus está

formado por dos genes estrechamente ligados que codifican dos tipos de subunidades (*x* e *y*) que difieren en su peso molecular (80-100 kDa) y, por tanto, presentan diferente movilidad en SDS-PAGE. Las subunidades de tipo *x*, muestran mayor peso molecular, mientras que las subunidades de tipo *y*, son más pequeñas (Harberd *et al.,* 1986). Sin embargo, ambas subunidades presentan la misma estructura: un péptido señal, un dominio N-terminal, un dominio repetitivo y un dominio C-terminal. La mayoría de las subunidades de tipo *x* poseen cuatro residuos de cisteína (tres en el dominio N-terminal y uno en el C-terminal), mientras que la mayoría de tipo *y* poseen siete residuos de cisteína (cinco en el dominio N-terminal, uno en el repetitivo y otro en el C-terminal) (Shewry *et al.,* 1995). El tamaño de los dominios N- y C-terminal suele estar muy conservado entre las diferentes HMW-GS, mientras la longitud del dominio repetitivo puede sufrir grandes variaciones, las cuales están asociadas a la variación alélica detectada en el dominio central (Shewry *et al.,* 1995).

Las LMW-GS por su parte, presentan pesos moleculares entre 30-50 kDa y están codificadas por los loci *Glu-A3*, *Glu-B3* y *Glu-D3*, situados en el brazo corto de los cromosomas del grupo homeólogo 1, el cual está estrechamente ligado con los loci *Gli-1* que codifican las γ- y ω- gliadinas (Liu, 1995; Pogna *et al.,* 1990; Singh and Shepherd, 1988). Las LMW-GS se dividen en B-, C- y D-LMW-GS en función de su movilidad en SDS-PAGE y su punto isoeléctrico (Jackson *et al.,* 1983). A su vez se dividen en subunidades siendo las B-LMW-GS las que presentan un mayor número de subunidades; la variabilidad de las

mismas está asociada a la calidad del trigo duro y a la elaboración de la pasta (Wrigley *et al.*, 2006). En función del primer aminoácido presente en la proteína madura, las LMW-GS también se pueden clasifican en tres subgrupos: LMW-I (isoleucina), LMW-M (metionina) y LMW-S (serina) (D'Ovidio y Masci, 2004). La estructura de estas subunidades consta de cuatro dominios: un péptido señal, un dominio N-terminal, un dominio repetitivo y un dominio C-terminal (formado por tres subdominios C-I, C-II y C-III). Sin embargo, las subunidades LMW-I carecen del dominio N-terminal, lo que da lugar a que el dominio repetitivo sea excluido en la formación de los puentes disulfuro intermoleculares del gluten (D'Ovidio and Masci, 2004).

En un principio, los estudios sobre la caracterización de las gluteninas LMW-GS debido a su complejidad multigénica eran más escasos que los de las gluteninas HMW-GS. Sin embargo, diferentes estudios han aportado nuevos datos que han permitido identificar los diferentes alelos que las conforman, No obstante, el número exacto de genes sigue siendo desconocido (Rasheed *et al.*, 2014).

La dureza del grano.

La dureza o textura del grano es el rasgo individual más importante que determina la calidad de uso final del trigo en el mercado mundial (Pomeranz and Williams, 1990). Se puede definir como el grado de adhesión entre las proteínas de la matriz y los gránulos de almidón del endospermo. La dureza determina, la cantidad

de almidón dañado que se produce durante el proceso de molienda en cada tipo de trigo. De acuerdo a esta característica, los trigos se clasifican en tres clases principales de dureza: muy duro, duro y blando. La clase muy dura se ha asociado exclusivamente al trigo duro, mientras que las otras dos se han encontrado en el trigo harinero (Morris and Rose, 1996). En el trigo harinero duro, la adhesión entre los gránulos de almidón y la matriz proteica en el endospermo es más fuerte que en el trigo harinero blando. Por tanto, la resistencia, la energía, el tiempo requerido y la cantidad de almidón dañado generado en la molienda son más elevados en los harineros duros que en los harineros blandos.

El almidón dañado está estrechamente relacionado con la absorción de la cantidad de agua (Guttieri *et al.,* 2001). Esto afecta el uso final de cada tipo de trigo ya que por un lado, el trigo harinero duro se utiliza para la elaboración de pan ya que requiere una alta absorción de agua para el correcto desarrollo de la masa fermentada y, por otro lado, el trigo harinero blando se utiliza para la elaboración de galletas y pasteles ya que el proceso requiere que haya más agua disponible para que el azúcar forme un jarabe lo cual se consigue con una harina menos hidrófila con partículas más finas y con menor cantidad de almidón dañado (Peña, 2002).

La dureza ha sido asociada a un complejo proteico denominado friabilina, el cual está controlado genéticamente por los genes *Pina-D1*, *Pinb-D1* y *Gsp-1* que codifican a PINA, PINB y GSP- 1, respectivamente (Hogg *et al.,* 2004; Morris, 2002; Morris *et al.,* 2013;

Morris *et al*., 1994). Los genes *Pina-D1* y *Pinb-D1* tienen una secuencia codificante de 447 pb (Gautier *et al.*, 1994) y sus secuencias son muy similares entre sí (70,2%) (Chantret *et al.*, 2005), mientras que el gen *Gsp-1* tiene una secuencia codificante de 495 pb (Morris *et al.*, 2013). Todos ellos están localizados en el locus de dureza (*Ha*) en el extremo distal del cromosoma 5D. Este locus es bastante complejo y está formado por 10 genes estrechamente ligados (Gautier *et al.*, 1994; Giroux and Morris, 1997; Morris, 2002; Rahman *et al.*, 1994; Wilkinson *et al.*, 2013).

Las puroindolinas son proteínas básicas, con un peso molecular en torno a los 13 kDa, ricas en cisteína, que se unen a los lípidos y constan de unos 148 aminoácidos (Morris, 2002). Estas proteínas presentan un esqueleto formado por 10 residuos de cisteína altamente conservado (Bhave and Morris, 2008) y un dominio rico en triptófano que consta de cinco residuos de triptófano en PINA y tres residuos en PINB (Pomeranz and Williams, 1990). Este dominio actúa como sitio de unión con los lípidos polares presentes en la membrana de los amiloplastos (Pauly *et al.*, 2013). Esta capacidad de unión a lípidos es la que determina su papel en la dureza del grano.

La dureza ha sido relacionada con la presencia/ausencia o modificaciones de algunos de los genes *Pin* (Bhave and Morris, 2008; Feiz *et al.*, 2009). Cuando ambos genes están en su forma silvestre (alelos *Pina-D1a* y *Pinb-D1a*) la textura del grano es blanda o suave, mientras que cuando están presentes mutaciones en los genes como cambios de nucleótidos en las regiones de codificación que afecten a

la proteína original o alguna deleción de los genes *Pin-D1* (alelos nulos) la textura del grano es dura (Bhave and Morris, 2008; Chen *et al.*, 2006; Gautier *et al.*, 1994; Giroux and Morris, 1997; Lillemo and Morris, 2000; Morris, 2002).

El caso extremo se produce en el trigo duro, ya que la alopoliploidización que dio lugar al trigo duro provocó la pérdida de los genes de las puroindolinas en los genomas A y B, por lo que el trigo duro carece de los genes de las puroindolinas, y sus granos son muy duros (Bhave and Morris, 2008; Gautier *et al.*, 2000; Li *et al.*, 2008). Sin embargo, la hibridación posterior con *Ae. tauschii* permitió la recuperación del carácter blando en los trigos hexaploides por la aportación de los dos genes procedentes del cromosoma 5D (Li *et al.*, 2008).

El papel del gen *Gsp-1* muestra cierta controversia ya que hay estudios que muestran que no está relacionado con la dureza (Tranquilli *et al.*, 2002) y otros que muestran cierto efecto en la misma (Gedye *et al.*, 2004). Si está confirmado su similitud con las PINs, que incluye la conservación del esqueleto de cisteínas y el dominio triptófano, aunque también existen algunas diferencias entre *Gsp-1* y las puroindolinas como la presencia en el N- terminal de 15 residuos asociados al péptido arabinogalactano (AGP), el cual forma parte de los polisacáridos no almidonados (Van den Bulck *et al.*, 2002). Sin embargo, estos péptidos pueden influir en la fuerza de adhesión entre los gránulos de almidón y la matriz proteica ya que se localizan en la membrana de los amiloplastos. Esto es importante ya que hay estudios

como el de Bettge and Morris (2000) que muestran que los polisacáridos no almidonados pueden influir hasta en un 76% en la variación de la dureza en trigos suaves, por tanto, *Gsp-1* podría tener un papel secundario en la dureza del trigo.

La búsqueda de nuevos alelos de estos genes es muy importante ya que el polimorfismo de los genes *Pin-D1* da lugar a diferencias en el grado de dureza y en los rasgos de calidad de procesamiento y uso final (Chen *et al.,* 2007), por tanto, aumentan el rango de texturas disponibles para así poder mejorar la calidad del trigo (Bhave and Morris, 2008). Tal es esto que, la búsqueda de nuevas variantes se ha extendido a especies relacionadas y un amplio rango de alelos y durezas ha sido detectado (Gautier *et al.,* 2000; Giroux *et al.,* 2000; Guzmán *et al.*, 2011; Lillemo *et al.,* 2002; Martin *et al.,* 2001; Simeone *et al.*, 2006).

El almidón y las proteínas waxy

El almidón es el principal carbohidrato de reserva sintetizado por las plantas superiores, tal es así, que en los granos de trigo el almidón representa aproximadamente el 70% de su peso seco (Hucl and Chibbar, 1996; Rahman *et al.,* 2000). Además, juega un importante efecto sobre la calidad de los productos alimentarios, ya que interviene en su estructura, y apariencia.

El almidón está formado por dos componentes estructurales diferentes: la amilosa (20-30% del total) y la amilopectina (70-80% del total) (Kuroda *et al.*, 1989). La amilosa es una molécula lineal formada

por residuos de D-glucosa unidos por enlaces α-1,4, mientras que la amilopectina es una molécula de cadena ramificada, formada por cadenas de D-glucosa unidas al igual que la amilosa por enlaces α-1,4, y las ramificaciones en enlaces α-1,6 cada 10-15 residuos (James *et al.,* 2003). La relación amilosa/amilopectina afecta a las propiedades fisicoquímicas del almidón, como la gelatinización, la pegajosidad y la gelificación (Zeng *et al.,* 1997); pero también puede afectar al valor nutricional, a la vida útil y a la calidad del uso final de diferentes productos de trigo (Hayakawa *et al.,* 2004; Regina *et al.,* 2006).

La síntesis del almidón se produce en el endospermo, concretamente dentro de un plastidio sin aparato fotosintético dedicado a esta función llamado amiloplasto. Esta síntesis comienza con el transporte de la glucosa-1-fosfato al amiloplasto. Una vez allí, varias enzimas la utilizan como sustrato actuando de forma secuencial formando así, la ruta de síntesis (Figura 4).

La ADP glucosa pirofosforilasa es la primera enzima que actúa, sintetizando la adenosina difosfato glucosa (ADPG) que será el sustrato para el resto de las enzimas almidón-sintasas, entre las que se encuentran: la almidón-sintasa unida al gránulo I (*GBSSI, Granule Bound Starch Synthase* I) o proteína waxy, la almidón-sintasa I (SSI, *Starch Synthase* I), la almidón-sintasa II (SSII, *Starch Synthase* II), la enzima ramificadora del almidón I (SBEII, *Starch Branching Enzyme*) y la enzima desramificante (DBE, *Debranching Enzyme*). De todas ellas, la proteína waxy es la única que se encarga exclusivamente de la

síntesis de amilosa, mientras que el resto de sintasas se encargan de la síntesis de amilopectina (Rahman *et al.*, 2000) (Figura 4).

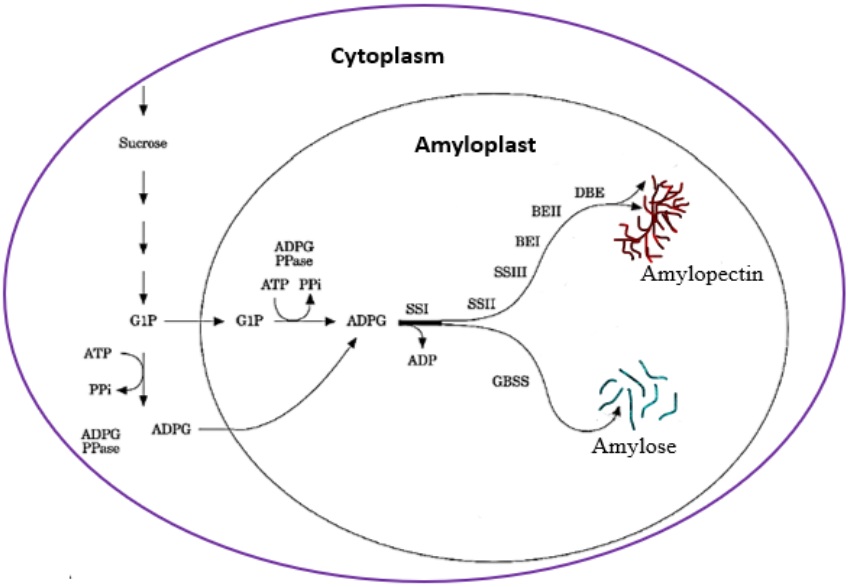


Figura 4. Síntesis del almidón en cereales (Rahman *et al.*, 2000)

En los últimos años las proteínas más estudiadas involucradas en la síntesis del almidón han sido las proteínas waxy, debido a su importancia en la composición del almidón. En trigo harinero hay tres proteínas waxy que son Wx-A1, Wx-B1 y Wx-D1 con un peso molecular de 60,1, 59,2 y 59,0 kDa, respectivamente (Fujita *et al.*, 1996), los cuales son sintetizadas por los genes *Wx (Wx-A1*, *Wx-B1* y *Wx-D1)* (Yamamori *et al.*, 1994). Estos genes están localizados en el brazo corto del grupo homólogo 7, excepto el gen *Wx-B1* que está localizado en el cromosoma 4AL debido a una translocación del cromosoma 7BS (Chao *et al.*, 1989; Yamamori and Endo, 1996).

Algunos estudios han mostrado que el nivel de variabilidad de estas proteínas es bajo, si lo comparamos con el de otras proteínas

como las proteínas de reserva del grano. Sin embargo, otros estudios han detectado cierta variabilidad en las proteínas waxy encontrando alelos con diferente punto isoeléctrico, diferente movilidad en SDS-PAGE e incluso alelos nulos (Guzmán and Alvarez, 2016). Éstos últimos son de gran importancia ya que pueden tener un gran impacto en la relación amilosa/amilopectina del almidón y por tanto en sus propiedades (Guzmán and Alvarez, 2016). Se han descrito diferentes tipos de alelos mediante la combinación de alelos salvajes y nulos en los loci *Wx-A1, Wx-B1* y *Wx-D1* llamados trigo parcialmente *waxy* (Yamamori *et al.,* 1994) y también trigos carentes de amilosa o trigos *waxy*. El primer trigo harinero *waxy* fue creado con el cruce del cv. Kanto 107 (*Wx-A1* y *Wx-B1* nulos) y del cv. Bai Huo (*Wx-D1* nulo) (Nakamura *et al.,* 1995). Sin embargo, debido a la presencia de tres loci *Wx* en el trigo hexaploide ha dificultado el desarrollo de trigos con bajo o nulo contenido de amilosa, (Yamamori and Quynh, 2000). En consecuencia, las líneas waxy parciales suelen mostrar contenidos de amilosa superiores al 16%, y sólo el trigo waxy (*Wx-A1b, Wx-B1b, Wx-D1b*) presenta valores bajos (inferiores al 3%) (Kim *et al.,* 2003; Miura *et al.,* 2002; Nakamura *et al.,* 1995). Por otro lado, la razón de los diferentes efectos de los alelos nulos es que cada una de las proteínas waxy tiene un nivel de expresión distinto (Yamamori and Quynh, 2000), siendo la proteína Wx-B1 la que más se expresa y tiene el efecto más significativo en la síntesis de amilosa (Kim et al., 2003).

El efecto de la variación del contenido de amilosa sobre las propiedades del almidón ha sido ampliamente estudiado (Araki *et al.,*

2000; Kim *et al*., 2003; Miura *et al.*, 2002; Wickramasinghe *et al.*, 2003). De forma que en la fabricación de alimentos saludables se utilizan las líneas de trigo con alto contenido en amilosa, ya que la amilosa se digiere más lentamente que la amilopectina, y la digestión lenta es beneficiosa para la salud humana (Behall and Scholfield, 2005). Por otra parte, las líneas de trigo que contienen menos amilosa o ninguna pueden ser importantes desde el punto de vista comercial ya que una disminución en el contenido en amilosa contribuye a retardar el enranciamiento de los panes, por tanto, prolonga la vida útil de diversos productos horneados (Lee *et al.*, 2001; Morita *et al.*, 2002). También para la fabricación de los tallarines orientales (*noodles*), se prefieren harinas de reducido contenido en amilosa para obtener almidones con mayor hinchamiento y menor firmeza que los de la pasta (Oda *et al.*, 1980; Peña, 2002). Por último, la calidad del almidón puede ser importante también fuera de la industria alimentaria, como en la generación de bioetanol a partir del grano, donde se ha demostrado un mayor rendimiento utilizando trigos *waxy* (Wu *et al.*, 2006).

Calidad nutricional del trigo

Los cereales constituyen la principal fuente de energía de la dieta. De hecho, se estima que el arroz, el maíz y el trigo representan alrededor del 60% de la energía que se obtiene de los alimentos a nivel mundial y, por tanto, constituyen un alimento básico para más de 7000 millones de personas (McKevith, 2004). Para el 40% de la población

mundial el trigo es el alimento básico, aportando un gran porcentaje de calorías, concretamente, entre el 20 y el 50% de la ingesta calórica total en países de clima templado. Sin embargo, el trigo es más que una fuente de calorías, ya que su grano está compuesto por carbohidratos (60-75%), proteínas (7-18%) y, lípidos (1,5- 2%), además de otros componentes importantes para la nutrición humana (Aykroyd y Doughty, 1970).

El hierro y el zinc son micronutrientes importantes en la nutrición humana. Ambos están presentes en el grano de trigo en cantidades significativas contribuyendo al 44 y al 25% de la ingesta diaria en los países desarrollados, respectivamente (Shewry, 2009). Esta cifra podría ser mayor en países en desarrollo donde millones de personas sufren cierto grado de carencia de micronutrientes, principalmente Fe y Zn. Esto ha llevado a que los programas de mejora incluyan estos micronutrientes en sus prioridades de mejora para paliar este problema (Bouis *et al.*, 2011).

La fibra alimentaria es otro componente importante ya que los productos derivados del trigo son una de las principales fuentes de este componente bioactivo (Pot *et al.*, 2012). Los principales tipos de fibras dietéticas son los β-glucanos y los arabinoxilanos (AX), siendo estos últimos los más abundantes en el trigo. Ambos tipos como en el caso de los micronutrientes tienen efectos diferentes en la salud humana y en la calidad del procesado y el uso final (Garófalo *et al.*, 2011). Los programas de mejora genética están empezando a incluir estos componentes del grano entre sus objetivos, con el fin de desarrollar

nuevos cultivares de trigo con mejores propiedades para la salud (Ibba *et al.,* 2021).

Por su parte, el gluten, además de ser responsable de las propiedades viscoelásticas de la masa, es responsable de una enfermedad llamada celiaquía, una afección autoinmune mediada por las células T que daña al revestimiento del intestino delgado. Los principales epítopos reactivos han sido identificados en las gliadinas, especialmente en las α-gliadinas (Rasheed *et al.,* 2014). Sin embargo, hay también algunos estudios que asocian las gluteninas con esta enfermedad debido a la presencia de epítopos reactivos similares a los encontrados en las gliadinas (Vader *et al.,* 2002)

Las mutaciones que afectan a la estructura de las proteínas de reserva pueden dar lugar a diferencias funcionales. Por lo tanto, la búsqueda de nuevas variantes de prolaminas es muy importante, ya que suponen una buena herramienta para la mejora de la calidad de la harina, con el objetivo de aumentar así el fondo genético del trigo. El papel de las prolaminas en la enfermedad celíaca hace necesario la selección de materiales menos reactivos con el fin de conseguir variedades aptas para celíacos. Por último, su elevado polimorfismo hace que sean muy buenas candidatas como marcadores de análisis de la diversidad genética.

Calidad de procesamiento y producto final.

Aunque el concepto de calidad parece muy sencillo, en realidad es un concepto complejo de evaluar ya que hay que medir numerosos

y diversos rasgos en función de las exigencias de los profesionales que trabajan con el trigo. Calidad puede ser definida de forma diferente según los distintos participantes de la cadena de valor del trigo (Breseghello and Sorrells, 2006):

- Para el agricultor la calidad de un trigo viene definida por obtener la máxima cantidad de grano y que éste tenga el máximo valor en el mercado.
- Para el molinero la calidad de un trigo viene determinada por cualidades como la textura y dureza del grano, el contenido en proteínas, el poder de absorción de agua y el peso específico. Todo esto afectará a la cantidad de harina que pueda obtener en la molienda.
- Para el panadero la calidad se manifiesta expresada por las condiciones plásticas de las harinas, de modo que produzcan masas elásticas y extensibles que permitan elaborar un producto con las características adecuadas.

Finalmente, aunque son numerosos los parámetros a estudiar a la hora de obtener un trigo que reúna estos caracteres de forma positiva, la evaluación de nuevos materiales debe responder a estas características. Para ello es importante el conocimiento de los procesos de análisis, de forma que los medios más utilizados para determinar la calidad en los trigos son los siguientes:

- Características de grano: peso hectolítrico, peso de mil granos, dureza, color, etc.

- Composición del grano: Proteínas (NIR, Dumas, Kjeldahl), cenizas, micronutrientes, pigmentos, etc.
- Propiedades reológicas de las harinas y las masas: farinógrafo, extensómetro, glucómetro, alveografo, mixografo, etc.
- Calidad de producto final: volumen de pan, calidad de la miga, firmeza de la pasta, etc.

Los recursos fitogenéticos

El comienzo de la Agricultura provocó una gran presión selectiva sobre las plantas cultivadas que dio lugar a una progresiva domesticación de muchas formas silvestres y a su adaptación a nuevos ambientes, manejos y usos (Cubero, 2003). En el caso del trigo, el hombre iba seleccionando las semillas que mejor se adaptaban a los usos pretendidos y esto provocó la generación de variedades locales con características agronómicas adaptadas a las zonas de cultivo. Esta práctica se ha mantenido hasta nuestros días y ha dado lugar a la sustitución de las muchas variedades locales por unas pocas variedades modernas. La mayoría de estas variedades han sido desarrolladas por programas de Mejora Genética en centros como el CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo) en el cual se han creado muchísimas de las variedades de trigo que hoy en día se cultivan en distintas partes del mundo. Este innovador proceso que se conoce como Revolución Verde, tuvo lugar en la década de los 60 del siglo XX, liderado por el ingeniero agrónomo Norman Borlaug y

permitió duplicar la producción mundial. Sin embargo, esta revolución está provocando una drástica reducción de la variabilidad genética del cultivo, debido al predominio cada vez mayor de una agricultura intensiva y a que solo una parte de la diversidad genética presente en las formas silvestres pasa a las formas cultivadas provocando un estrechamiento de la base genética, hasta el punto de hacer prácticamente desaparecer las variedades locales usadas en la agricultura tradicional durante siglos (Esquinas-Alcázar, 2005).

La reducción de la variabilidad genética ha provocado una deriva muy importante de genes, por lo que una gran parte de la variabilidad genética existente para diferentes genes podría haber desaparecido de los campos de cultivos (Hammer, 2003). La diversidad genética de los individuos resulta fundamental para la supervivencia de la especie a largo plazo. Si ella se reduce en una población, disminuye también su capacidad de adaptarse ante potenciales cambios ambientales, incrementado la vulnerabilidad de los cultivos debido a la pérdida de genes de interés necesarios para enfrentarse a plagas y/o a nuevas enfermedades.

Para poder recuperar parte de esta variabilidad genética pérdida, hay dos vías. Una de ellas es la llamada conservación ex-situ que consiste en la conservación en Bancos de germoplasma de plantas vivas, semillas, ADN y polen (Hammer *et al.*, 2001) y la otra opción es la conservación cruzada que consiste en la protección de los ecosistemas a través del mantenimiento y conservación de variedades locales mediante técnicas tradicionales por parte de los propios

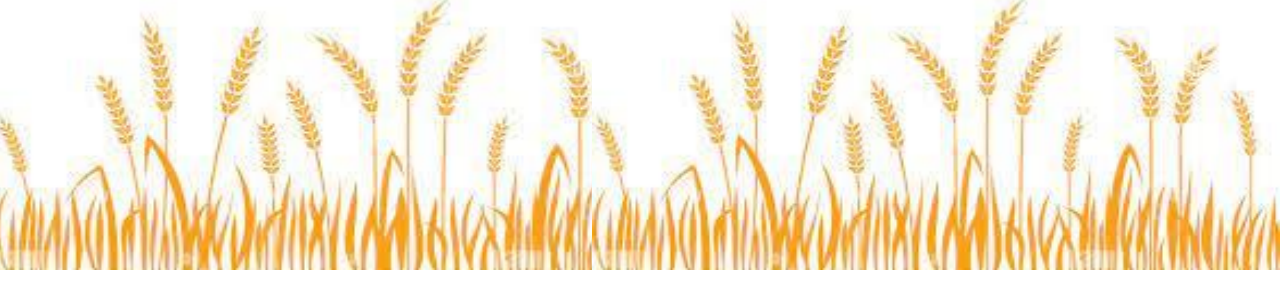
agricultores. Este tipo de conservación ha sido mayoritariamente asociada a plantas de escaso interés agrícola durante mucho tiempo. Sin embargo, a partir del Tratado Internacional sobre los Recursos Fitogenéticos para la Alimentación y la Agricultura (FAO, 2001), este tipo de conservación se ha puesto en auge. Ambos métodos son complementarios y el uso simultáneo de ellos podría asegurar la correcta conservación de los recursos genéticos, aumentando así, la variabilidad genética.

Actualmente el cultivo del trigo en muchos países depende de un grupo de cultivares muy relacionados genéticamente. Esto es un problema, por lo que existe una necesidad de buscar otros materiales que permitan aumentar la base genética del cultivo y que además sean donantes potenciales de genes útiles. Esto ha hecho que se esté produciendo un resurgimiento de los cultivos de trigos antiguos infrautilizados o abandonados, entre los que se encuentran el espelta. Estos cultivares antiguos podrían constituir una fuente importante de variación para caracteres interés como caracteres relacionados con la calidad del trigo, resistencia a enfermedades y a situaciones adversas (Alvarez and Guzmán, 2018; Zaharieva and Monneveux, 2014)

Nuestro grupo de investigación ya ha realizado diversos estudios sobre la mayoría de las características de calidad mencionadas en una amplia colección de materiales, procedentes de diferentes bancos de Germoplasma detectándose una importante variación que puede ser usada en la mejora del trigo moderno o en la recuperación de antiguos cultivos (Alvarez and Guzmán, 2012). No obstante, una parte

destacada de los recursos genéticos de trigo está aún sin explorar, por lo que se da la necesidad de caracterizar la variación para los componentes relacionados con calidad en otras colecciones de trigos antiguos de otros países de las que se dispone, para su posible uso en programas de mejora.

OBJETIVOS



En esta Tesis Doctoral se ha evaluado y caracterizado la variabilidad genética para los genes y componentes implicados en la calidad del trigo en recursos genéticos procedentes de diversas regiones del mundo. Para ello se han planteado los siguientes objetivos:

1) Analizar la variación en los loci *Glu-A^{m}1*, *Wx-A^{m}1* y *Ha* en una colección de 170 accesiones de einkorn de Irak, Irán y Turquía, con el fin de establecer el valor potencial de esta especie silvestre como fuente donante de genes para la mejora de la calidad del trigo.

2) Evaluar la diversidad de los genes *Pina-D1* y *Pinb-D1*, en una colección de 271 variedades locales (*landraces*) de trigo harinero de Irán, con el fin de detectar nuevos alelos de los genes de las puroindolinas asociados a diferentes durezas del grano.

3) Analizar la variabilidad de diferentes componentes del grano con importancia nutricional en una colección española de trigo espelta y variedades modernas de trigo harinero. Asimismo, identificar genotipos que puedan utilizarse en programas de mejora para desarrollar nuevos cultivares adaptados de alto rendimiento y calidad nutricional.

4) Evaluar una colección española de trigo espelta y variedades modernas de trigo harinero para características de calidad de grano relacionadas con la calidad tecnológica.

CAPÍTULO I

Potential use of wild einkorn wheat for wheat grain quality improvement: Evaluation and characterization of *Glu-1*, *Wx* and *Ha* loci.

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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

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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

Resumen

La escaña silvestre *(Triticum monococcum* L. ssp. *aegilopoides* (Link) Thell.) es una especie de trigo diploide de Oriente Próximo que se ha clasificado como ancestro del primer trigo cultivado *(*escaña*; T. monococcum* L. ssp. *monococcum)*. Su genoma (A^m), aunque no es el donante del genoma A del trigo poliploide, muestra una gran similitud con el genoma A^u. Una característica importante para la mejora del trigo es la calidad del grano, que está asociada a tres componentes del trigo: las proteínas de almacenamiento del endospermo (propiedades del gluten), las sintasas del almidón (características del almidón) y las puroindolinas (dureza del grano). En el presente estudio, se estudió la variabilidad para estas características de calidad del grano en una colección de escaña silvestre. El uso combinado de análisis de proteínas y de ADN permitió detectar numerosas variantes para cada uno de los siguientes genes: seis para *Ax*, siete para *Ay*, ocho para *Wx*, cuatro para *Gsp-1*, dos para *Pina* y tres para *Pinb*. La elevada presencia de variantes en esta especie sugiere su potencial como fuente de nuevos alelos que podrían utilizarse en la mejora genética del trigo moderno.

Palabras clave: Trigo diploide; Recursos genéticos; Puroindolinas; Proteínas de almacenamiento del grano; Proteína waxy

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

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 (Alvarez *et al.,* 2010). At the same time, global warming has become one of the main challenges facing crop improvement programs (Newton *et al.,* 2011; Curtis *et al.,* 2014). 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) (Wrigley *et al.,* 2006) the starch synthases affect the composition and properties of the starch (Guzmán *et al.,* 2016) 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 (Morris, 2002). 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 (Wrigley *et al.*, 2006; Guzmán and Alvarez, 2016; Morris, 2002; Demeke *et al.*, 1997; D'Ovidio *et al.*, 2004; Graybosch *et al.*, 1998; Shewry *et al.*, 1992). 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 (Payne, 1987) 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) (Singh and Shepherd, 1988).

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 (Wrigley *et al.*, 2006). 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 (Guzmán and Alvarez, 2016) 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 (Li *et al.*, 2007). 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, (Li *et al.*, 2007) 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 (Srivastava and Damania, 1989), 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 (Alvarez and Guzmán, 2018). 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 (Johnson and Dhaliwal, 1976; Sharma and Waines, 1981). 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*) was domesticated, the A genome of polyploid wheats is equivalent to that of *T. urartu* (Dvorak *et al.*, 1988).

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 (Hidalgo *et al.*, 2013; Hidalgo and Brandolini, 2014; Arzani and Ashraf, 2017; Brandolini *et al.*, 2018; Hidalgo *et al*., 2019; Geisslitz *et al.*, 2019; Malalgoda *et al.*, 2019). As the decline in this crop began in antiquity (Zohary and Hopf, 2000; Zaharieva and Monneveux, 2014; Arranz-Otaegui *et al.*, 2018), 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 (Alvarez and Guzmán, 2018) 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 (Zamir, 2001), 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 (Dong *et al*., 2017; Cseh *et al.*, 2019).

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.

Materials and Methods

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).

Glutenin Analysis

Proteins were extracted from crushed endosperm according with the procedure described by Alvarez *et al.* (2001). 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.

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 (1981). 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.

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 (Stacey and Isaac, 1994)

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.* (1995). 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 (2012) and Ayala *et al*. (2015): 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.* (2004) 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.* (2006).

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.

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 (Lafiandra *et al.*, 1997; Alvarez *et al.*, 2013; Ayala *et al.*, 2013; Alvarez *et al.*, 2013; Alvarez *et al.*, 2021): *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.

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.

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 (Waines and Payne, 1987; Saponaro *et al.*, 1995).

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).

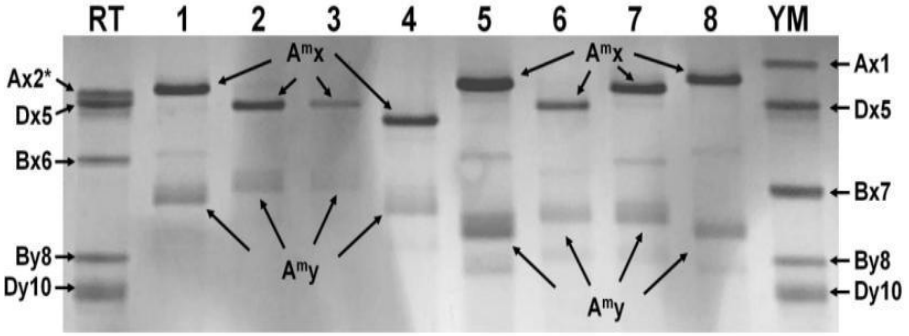


Figure 1. SDS-PAGE of representative variation for HMWGs found in the wild einkorn collection. Lanes are as follows: **RT,** common wheat cv. Rota; **1,** PI 554559; **2,** PI 427622; **3,** PI 427623; **4,** PI 554504; **5,** PI 554548; **6,** PI 427453; **7,** PI 554521; **8,** PI 470720; and **YM,**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

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 $A^{m}x$ and $A^{m}y$ 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 (Lafiandra *et al.*, 1997; Alvarez *et al.*, 2013; Ayala *et al.*, 2013; Alvarez *et al.*, 2013). In these accessions, both $A^{m}x$ and $A^{m}y$ 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 (Lafiandra *et al.*, 1997; Alvarez *et al.*, 2013). 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 these three fragments are located inside the central repetitive domain.

The cleavage patterns of the Ax2* amplicon from cv. Cheyenne (Figure 2, lane 1) and the *A^{m}x* amplicons detected in wild einkorn were clearly different. The *A^{m}x* patterns were more similar to the Ax1 or Axnull amplicons, as shown by Alvarez *et al.* (2013), 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 (Alvarez *et al.*, 2013) 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 *A^{m}x* 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.

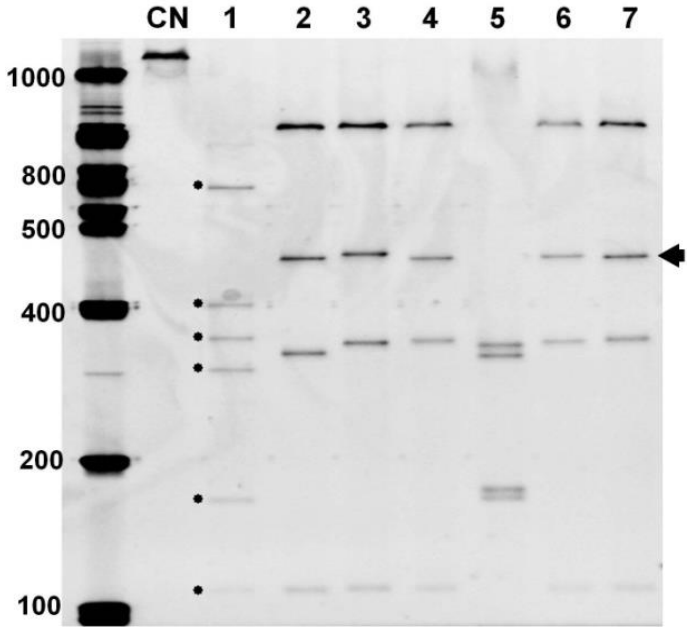


Figure 2. DNA digestion of PCR products from *Amx* alleles of the materials evaluated using *Hae*III endonuclease. The band size of the molecular weight marker is expressed in bp. Lanes are as follows: **1**, common wheat cv. Cheyenne; **2,** PI 427498; **3,** PI 427497; **4,** PI 427622; **5,** PI 554504; **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.

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). 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 (D'Ovidio *et al.,* 1996). 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).

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

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

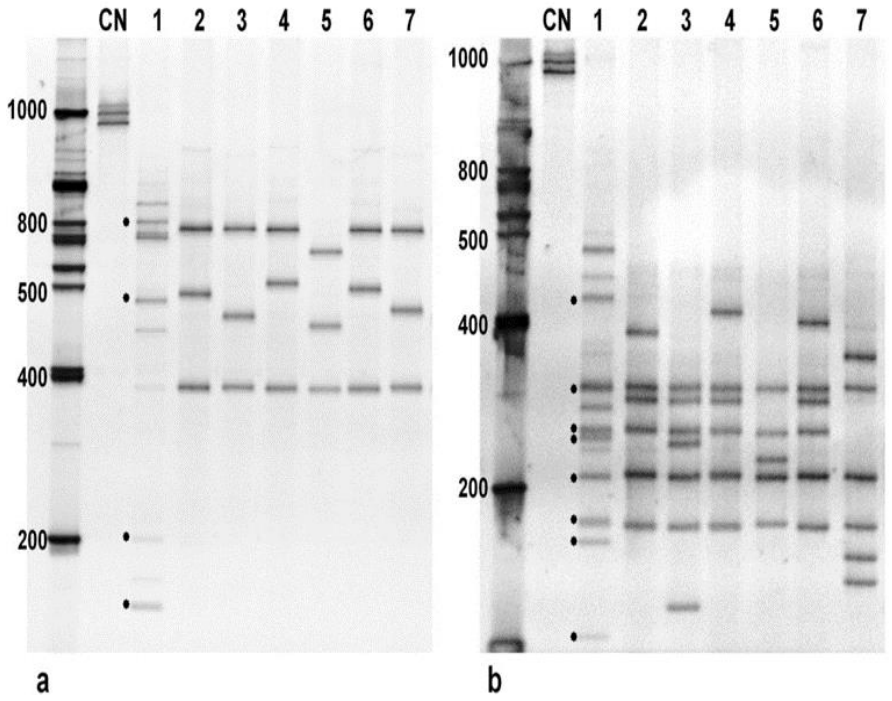


Figure 3. DNA digestion of PCR products from $A^{m}y$ alleles of the materials evaluated using *Dde*I (**a**) and *Pst*I (**b**) endonucleases. The band size of the molecular weight marker is expressed in bp. Lanes are as follows: **1**, common wheat cv. Cheyenne; **2,** PI 427498; **3,** PI 427497; **4,** PI 427622; **5,** PI554504; **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.

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).

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 (Guzmán and Alvarez, 2012; Ayala *et al.*, 2015; Alvarez *et al.*, 2021), 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-A^{m}1* variants presented one restriction pattern very different to the reference pat- tern, 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.

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).

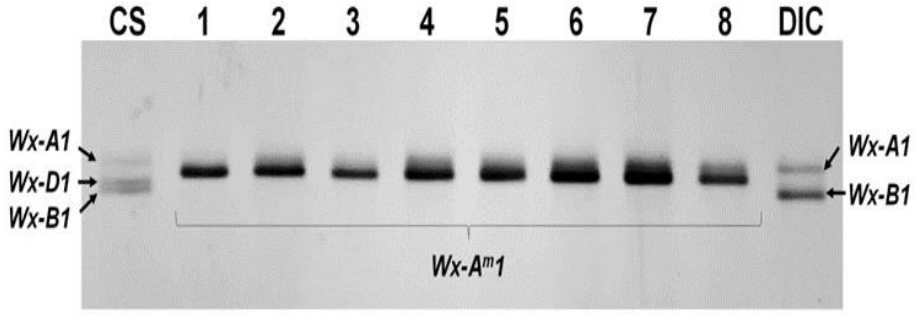


Figure 4. SDS-PAGE of representative variation for waxy proteins found in the wild einkorn collection. Lanes are as fol- lows: CS, common wheat cv. Chinese Spring; 1, PI 554559; 2, PI 427622; 3, PI 554504; 4, PI 554548; 5, PI 427453; 6, PI 470720; 7, PI 470713; 8, PI 427575; and DIC, emmer wheat landrace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

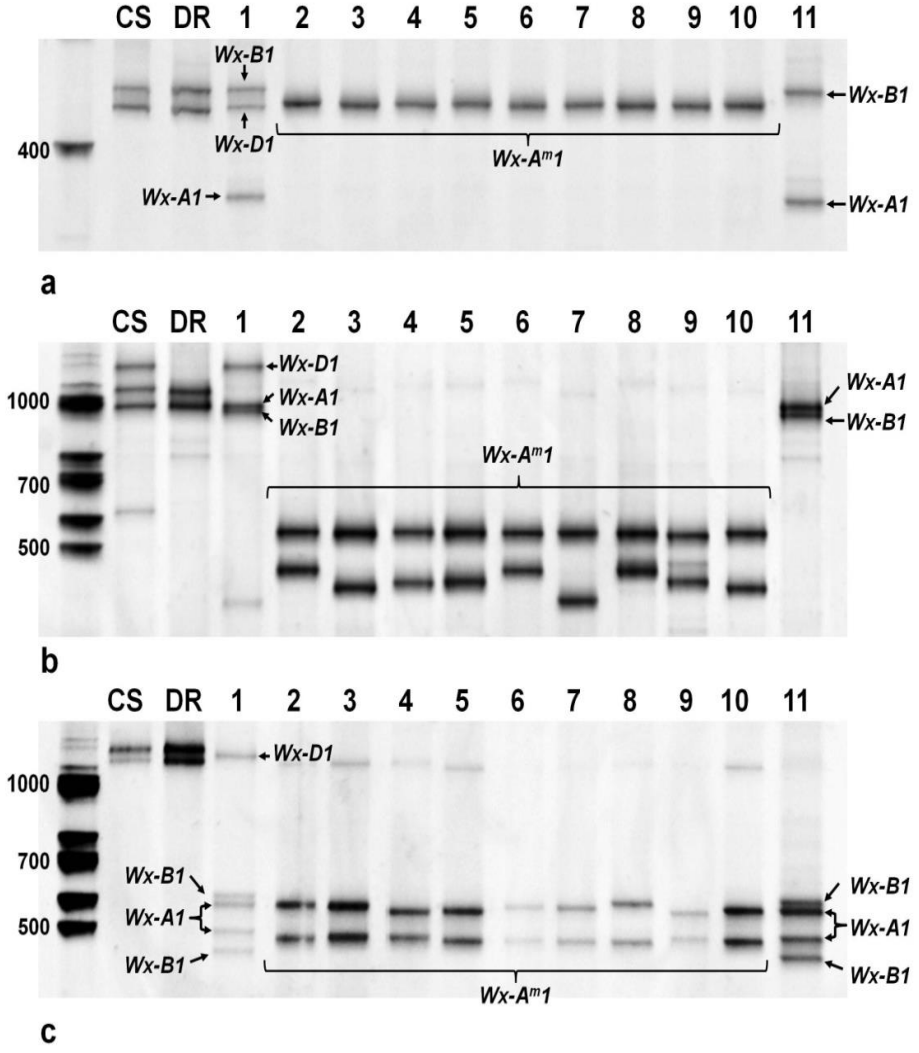
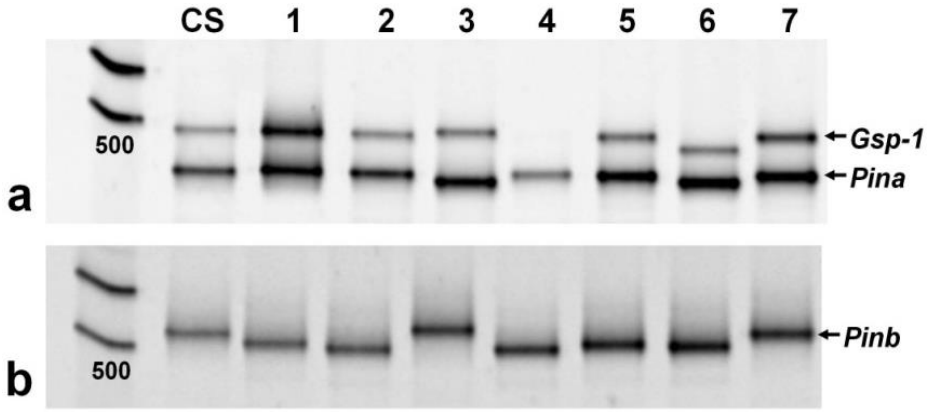


Figure 5. DNA digestion of PCR products from *Wx* alleles of the materials evaluated: (**a**) Wx1Fw/Wx1.3Rv fragment with *Dde*I; (**b**) Wx2Fw/Rv fragment with *Dde*I; and (**c**) Wx3Fw/Rv frag- ment with *Nco*I. Lanes are as follows: **1**, common wheat cv. Chinese Spring; **2,** PI 554559; **3,** 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.

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 de- signed by Massa *et al.* (2004) and Lillemo *et al.* (2006) (Figure 6a and 6b, respectively).



The Massa *et al.* (2004) 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 ampli- cons 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 (Ayala *et al.,* 2013; Guzmán *et al.,* 2012).

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.

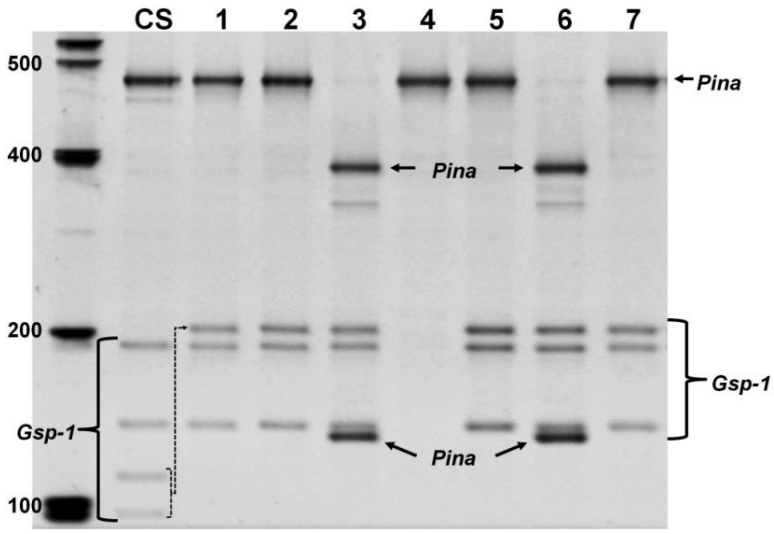


Figure 7. DNA digestion with *Dde*I of PCR products from *Gsp-1* and *Pina* gene alleles of the materials evaluated. Lanes are as follows: **CS**, common wheat cv. Chinese Spring; **1**, PI 554559; **2**, PI 427622; **3**, PI 554504; **4**, PI 554548; **5**, PI 427453; **6**, PI 470720; and **7**, PI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

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 (Hajjar and Hodgkin, 2007; Schneider *et al.*, 2008; Gill *et al.*, 2011). However, the role of the primary wheat gene pool, such as the accessions studied here, in grain quality improvement has been more limited (Alvarez and Guzmán, 2018). 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 (Mason-Gamer *et al.*, 1998).

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 (Caballero *et al.*, 2008; Caballero *et al.*, 2009), and was clearly greater than the variation shown in some of the studies carried out with cultivated wheat species. For example, Alvarez *et al.* (2006), using Spanish cultivated einkorn materials, detected only three *Glu-Am1* 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 (Arranz-Otaegui *et al.,* 2018). 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 (Zaharieva and Monneveux, 2014; Arranz-Otaegui *et al.,* 2018). 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 (Lawrence *et al.,* 1988; Law and Payne, 1983); 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*. (1997) 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 (Alvarez *et al.,* 2009). 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.* (2014) analyzed the genomic sequences of the *Wx-A1* genes, detecting up to five alleles in *T. urartu*, one of which had previously been described Guzmán *et al.* (2012), but only one of which was found in the cultivated einkorn accessions analyzed, confirming the findings of Guzmán *et al.* (2009). In general, the studies carried out on this gene in cultivated einkorn have shown that variation is scarce (Urbano *et al.*, 2002; Rodriguez-Quijano *et al.*, 2004).

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 (Morris, 2002; Morris *et al.*, 2013), and the true function of the seven other genes is unknown (Li *et al.*, 2008). While the diversity of puroindoline genes has been evaluated (Guzmán *et al.*, 2012; Lillemo *et al.*, 2002; Simeone *et al.*, 2006; Chen *et al.*, 2009), 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* (Chen *et al.*, 2009).

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 (Li *et al.*, 2008).

One clear opportunity to increase genetic variability is by reincorporating 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 $5A^{m}S$ chromosome, was transferred to cv. Chinese Spring, as either an entire chromosome (Luo *et al.*, 2000; Tranquilli *et al.*, 2002) or only the *Ha* locus (Bonafede *et al.*, 2007), 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 (Morris *et al.*, 2011). 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.* (1995) 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 in- version 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* (Upadhya, 1963). A similar occurrence was also reported by Gill *et al.* (1988) in crosses between wild einkorn and durum wheats, which have shown potential for breeding (Megyeri *et al.,* 2011). In our previous work using *T. urartu*, several backcrosses were achieved between the hybrid and the durum wheat parent (Alvarez *et al.,* 2009).

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.

Conclusion

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.

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CAPÍTULO II

Allelic variation of puroindolines genes in Iranian common wheat landraces.

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Allelic Variation of Puroindolines Genes in Iranian Common Wheat Landraces

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Abstract: Wheat is one of the most widely grown crops in the world. One of the traits that defines wheat quality is grain hardness, which is determined by puroindolines (PINA and PINB) proteins encoded with *Pina-D1* and *Pinb-D1* genes. In this study, the diversity of *Pina-D1* and *Pinb-D1* was evaluated in a collection of 271 Iranian common wheat (*Triticum aestivum* L. ssp. *aestivum*) landraces, whose kernels had previously been classified as hard or semi-hard based on PSI analysis. Three alleles previously described as associated with hard grain were detected in the collection: *Pinb-D1b* in 11 accessions, *Pinb-d1ab* in 175 accessions, and *Pinb-d1p* in 80 accessions. In addition, a novel allele tentatively named *Pinb-d1ak* was detected in *Pinb-D1* and was characterized by a change at position 140 of the deduced protein (cysteine/tyrosine). On average, the accessions with this allele showed a lower PSI value than the accessions with other *Pin* allele. This means that this novel allele may be associated with harder grains than other *Pin* alleles and could be used by breeding programs targeting different grain hardness levels. This study highlights the importance of conserving and characterizing wheat genetic resources that could be used as sources of genetic variability in breeding programs.

Keywords: wheat landraces; *Triticum aestivum*; grain hardness; puroindolines; genetic diversity

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1. Introduction

Wheat (*Triticum* sp.) is one of the three main crops grown around the world, with a

Resumen

El trigo es uno de los cultivos más extendidos en el mundo. Uno de los rasgos que definen la calidad del trigo es la dureza del grano, que viene determinada por las proteínas puroindolinas (PINA y PINB) codificadas por los genes *Pina-D1* y *Pinb-D1*. En este estudio se evaluó la diversidad de *Pina-D1* y *Pinb-D1* en una colección de 271 variedades locales de trigo blando iraní (*Triticum aestivum* L. ssp. *aestivum*), cuyos granos se habían clasificado previamente como duros o semiduros en función del análisis de PSI. En la colección se detectaron tres alelos previamente descritos como asociados a los granos duros: *Pinb-D1b* en 11 accesiones, *Pinb-D1ab* en 175 accesiones y *Pinb-D1p* en 80 accesiones. Además, se detectó un nuevo alelo en *Pinb-D1* denominado provisionalmente *Pinb-D1ak* que se caracterizó por un cambio en la posición 140 de la proteína deducida (cisteína/tirosina). La media de las accesiones con este alelo mostró un valor PSI más bajo que las accesiones con otros alelos *Pin*. Esto significa que este nuevo alelo puede estar asociado con granos más duros que otros alelos *Pin* y podría ser utilizado por los programas de mejora dirigidos a diferentes niveles de dureza del grano. Este estudio pone de manifiesto la importancia de conservar y caracterizar los recursos genéticos del trigo que podrían utilizarse como fuentes de variabilidad genética en los programas de mejora.

Palabras clave: Variedades locales de trigo; *Triticum aestivum*; Dureza del grano; Puroindolinas; Diversidad genética

Abstract

Wheat is one of the most widely grown crops in the world. One of the traits that defines wheat quality is grain hardness, which is determined by puroindolines (PINA and PINB) proteins encoded with *Pina-D1* and *Pinb-D1* genes. In this study, the diversity of *Pina-D1* and *Pinb-D1* was evaluated in a collection of 271 Iranian common wheat (*Triticum aestivum* L. ssp. *aestivum*) landraces, whose kernels had previously been classified as hard or semi-hard based on PSI analysis. Three alleles previously described as associated with hard grain were detected in the collection: *Pinb-D1b* in 11 accessions, *Pinb-d1ab* in 175 accessions, and *Pinb-d1p* in 80 accessions. In addition, a novel allele tentatively named *Pinb-D1ak* was detected in *Pinb-D1* and was characterized by a change at position 140 of the deduced protein (cysteine/tyrosine). On average, the accessions with this allele showed a lower PSI value than the accessions with other *Pin* allele. This means that this novel allele may be associated with harder grains than other *Pin* alleles and could be used by breeding programs targeting different grain hardness levels. This study highlights the importance of conserving and characterizing wheat genetic resources that could be used as sources of genetic variability in breeding programs.

Keywords: Wheat landraces; *Triticum aestivum*; Grain hardness; Puroindolines; Genetic diversity

Introduction

Wheat (*Triticum* sp.) is one of the three main crops grown around the world, with a field area of 219 million hectares that represents a quarter of total cereal production, with 760 million tons produced in 2020 (FAOSTAT, 2020).

In wheat, grain quality is one important factor because it determines the market value and its subsequent use. Wheat quality is determined by grain composition and the characteristics derived from it: hardness, gluten strength, and starch. Grain hardness is the most important single trait determining end-use quality (Pomeranz and Williams, 1990), as it is related to the amount of damaged starch produced during the milling process. Since hard grain produces more damaged starch than soft grain, hard wheat is used for bread-making, which requires high water absorption for the correct development of fermented dough, while soft wheat is preferred for making cookies and pastries, which requires less hydrophilic flour, so that more water is available for the sugar to form a syrup, resulting in greater cookie spread (Guttieri *et al.*, 2001).

In common wheat (*Triticum aestivum* L. ssp. *aestivum*), grain hardness is genetically controlled by two genes (*Pina-D1* and *Pinb-D1*) that codify for puroindolines (PINA and PINB, respectively). Puroindolines are small (~13 kDa), cysteine-rich, lipid-binding proteins consisting of 148 amino acids (Morris, 2002). These proteins exhibit a tryptophan-rich domain, which consists of five tryptophan residues in PINA and three residues in PINB (Pomeranz and Williams, 1990), and

both PINA and PINB contain a backbone of 10 cysteine residues (Bhave and Morris, 2008). The *Pin-D1* genes are located at the hardness locus (*Ha*) in the distal end of the 5DS chromosome (Giroux and Morris, 1997). Both genes have a 447 bp coding sequence (Gautier *et al.,* 1994), and these sequences are 70.2% similar to each other (Chantret *et al.*, 2005). The *Ha* locus includes also the *Gsp-1* gene (495 bp) that codes for the grain softness protein-1 (Morris *et al.,* 2013).

The presence of *Pina-D1a* and *Pinb-D1a* alleles in common wheat is associated with a soft texture, while nucleotide changes in coding regions affecting the original protein or deletions of entire *Pin-D1* genes (null alleles) are correlated with a hard texture (Bhave and Morris, 2008). Since allopolyploidization resulting in durum wheat (*T. turgidum* ssp. *durum* (Desf.) Husn) caused the loss of the puroindolines genes in the A and B genomes, durum wheat lacks puroindolines genes, and its grains are very hard (Bhave and Morris, 2008). The *Pin-D1* genes' polymorphisms led to differences in the degree of hardness and in processing and end-use quality traits (Chen *et al.*, 2007). Several studies were conducted comparing the effects of the *Pina-D1b* and *Pinb-D1b* alleles, which are the two most common *Pin-D1* alleles with hard endosperm. Genotypes with *Pina-D1b*/*Pinb-D1a* showed harder kernels than those with *Pina-D1a/Pinb-D1b* (Giroux *et al.,* 2000). In addition to this, the *Pinb-D1b* allele was associated with higher milling yield, higher dough extensibility, and better baking quality (higher loaf volume and better crumb) than the *Pina-D1b* allele (Martin *et al.,* 2001). These studies clearly illustrate the importance of the *Pin-D1* genes not only for

hardness but also for processing and end-use quality.

Wheat landraces possess variability in different genes that are not present in modern cultivars (Hammer *et al.,* 1996). For wheat quality improvement, this variability could be useful in generating materials with novel properties. In fact, several puroindoline alleles have been found in different collections of genetic resources worldwide. For example, the allele *Pinb-D1d* was first detected when researchers were analyzing *Pin-D1* genes of cultivars from northern Europe (Lillemo and Morris, 2000), and later the same allele was found in cultivars with Spanish origins (Ayala *et al.,* 2013). Chinese landraces have been showed to carry alleles very rare in other germplasm pools, such as *Pina-D1r*, *Pina-D1s,* and *Pinb-D1p* (Ma *et al.,* 2017) or *Pinb-D1l* (Tanaka *et al.,* 2008). Similarly, cultivars with origins in India were found to be good sources for the variability of the *Pin-D1* genes by Kumar *et al.* (2015), who identified five novel alleles in materials from that country.

Iran is one of the main habitats of wheat ancestors and is therefore a reservoir of new alleles (Sansaloni *et al.,* 2020). In the Seeds of Discovery project at CIMMYT (International Maize and Wheat Improvement Center, Mexico), 30,000 wheat accessions (including a collection of 6800 Iranian wheat landrace accessions (Sansaloni *et al.,* 2020) were characterized for quality traits (Vikram *et al.,* 2021), including grain hardness, using the PSI method (AACC, 2000).

The objective of our study was to assess the diversity for the *Pina-D1* and *Pinb-D1*genes in a collection of 271 common wheat landraces from Iran, in order to detect new alleles of the puroindoline genes

associated with different grain hardnesses.

Material and Methods

Plant Materials

In this study, 271 Iranian common wheat landraces, which were provided by the CIMMYT's germplasm bank and known from a previous study to have hard and semi- hard grains, were used (Vikram *et al.*, 2021). The hardness data of all of them were determined by near infrared spectroscopy (NIRS, Antaris II FT-Analyzer, Thermo Scientific, Waltham, MA, USA), calibrated on the basis of AACC methods (AACC, 2000) (Supplementary Table S1).

Genomic DNA Extraction

Genomic DNA was extracted from the young leaves of approximately two-week-old seedlings grown using the modified CTAB method described by Stacey and Isaac (1994), and the samples were diluted to a final concentration of 20 ng/mL.

Amplification and Digestion of Pina-D1 and Pinb-D1 Genes

The *Pina-D1* gene was amplified with the primers designed by Lillemo *et al.* (2006) (LIL1-Fw:5′-CATCTATTCATCTCCACCTGC-3′ and LIL1-Rv:5′-GTGACAGTTTATTAGCTAGTC-3′) and with the primers designed by Massa *et al.* (2004) (MAS-Fw: 5′-GGTGTGGCCTCATCTCATCT-3′ and MAS-Rv: 5′-

AAATGGAAGCTACATCACC AGT-3′). For the amplification of the *Pinb-D1* gene, we used the primers designed by Lillemo *et al.* (2006) (LIL2-Fw: 5′- GAGCCTCAACCCATCTATTCATC-3′ and LIL2-Rv: 5′-CAAGGGTGATTTT ATTCATAG-3′).

Each 15 mL reaction included 40 ng of DNA, 1.5 mM $MgCl_2$, 0.3 mM of each primer, 0.2 mM dNTPs, 3 µL of 5x PCR buffer, and 0.75 U of GoTaq® G2 Flexi DNA polymerase (Promega). The PCR conditions included an initial denaturation for 3 min at 94 °C followed by 35 subsequent cycles: 45 s at 94 °C, 1 min 30 s at 60 °C, and then 45 s at 72 °C for the primers LIL1 and LIL2; and 45 s at 94 °C, 30 s at 64 °C, and then 45 s at 72 °C for the primer MAS. After the 35 cycles, all reactions included a final extension of 5 min at 72 °C. The amplification products were fractionated on vertical PAGE gels in a discontinuous Tris-HCl buffer system (pH 6.8/8.8) with 8% (*w/v*, C: 1.28%) polyacrylamide concentration.

To detect some mutations already described in *Pina-D1* and *Pinb-D1* genes, the amplicons of these genes were digested with endonucleases or restriction enzymes. The *Pina-D1* amplicons were digested with *Msp*I (Biolabs) to detect the *Pina-D1m* allele (Chen *et al.*, 2006) and with *Dde*I (Biolabs) to see if there were differences in the pattern when digesting the different accessions. The *Pinb-D1* amplicons were digested with *Bsr*BI (Biolabs) to detect the *Pinb- D1b* allele (Wang *et al.*, 2008), with *Pvu*II (Biolabs) to detect the *Pinb-D1c* allele (Lillemo and Morris, 2000), with *Bst*XI (Biolabs) to detect the *Pinb-D1e* allele (Wang *et al.*, 2008), with *Apa*I (Biolabs) to detect the *Pinb-d1ab* allele

(Wang *et al*., 2008), and with *Pfl*MI (Biolabs) to detect the *Pinb-D1p* allele (Li *et al.*, 2008). Digests were performed in a total volume of 10 µL, with 1 µL of buffer (1x), 0.25 U of enzyme, and 5 µL of PCR product for 2 h at 37 °C. The products resulting from this digestion were analyzed using electrophoresis on 10% polyacrylamide concentration gels (*w/v*; C: 3%).

Cloning of Pinb-D1 Genes

The *Pinb-D1* amplicons were then purified with SureClean Plus (Bioline Reagents, London, UK) and cloned into the vector pSpark TA Done (Canvax, Cordoba, Spain). The nucleotide sequences were obtained from three positive clones. These sequences were analyzed and compared with the common wheat sequences available in the NCBI database for *Pinb-D1a*: DQ363913 using the Geneious Pro software version 2020.2.4 (Biomatters Ltd., Auckland, New Zealand). To evaluate the impact of the allelic variations in the PINB protein's function, the potential effects of amino acid changes were analyzed with PROVEAN (Protein Variation Effect Analyzer) (Choi *et al.*, 2012). In PROVEAN (http://provean.jcvi.org, accesed on 1 June 2022), variants with values below -2.5 are predicted as deleterious, while values above -2.5 are considered neutral (Choi *et al.*, 2012). Finally, the novel sequence found in the current study is available from the Genbank database (NCBI ID: ON723901).

Results

Variation in Grain Hardness

A large set of Iranian common wheat landraces were evaluated previously for grain hardness using the PSI test (Vikram *et al.,* 2021). For the current study, a subset of those accessions was selected based on their PSI values. The selected accessions showed an average PSI value of 47.14 ± 2.74% and values ranging between 40 and 50%. These values correspond to those of hard and semi-hard grains: 221 accessions showed a semi-hard grain texture ranging between 45 and 50%, while 50 accessions were classified as semi-hard grain texture since they ranged between 45 and 40% (Figure 1). According to the literature (Bhave and Morris, 2008), these accessions should carry mutations in either *Pina-D1* or *Pinb-D1* that lead to their non-soft grain texture.

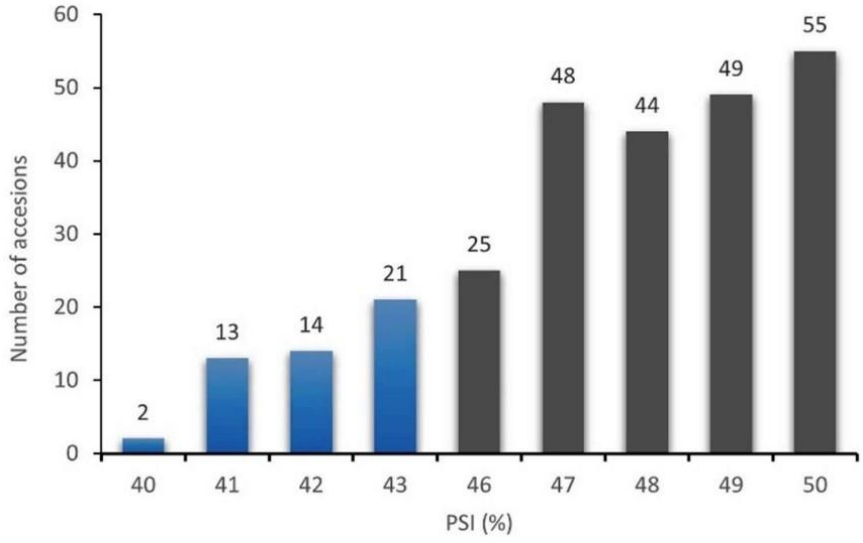

Figure 1. PSI frequency distribution of the accessions evaluated. Blue bars indicate hard grain accessions; grey bars correspond to semi-hard grain accessions.

PCR Analysis of the Pina-D1 and Pinb-D1 Genes

The *Pina-D1* and *Pinb-D1* genes were amplified to detect possible differences in their size, together with the presence/absence of amplicons. For the *Pina-D1* gene using primer LIL1, all of the accessions produced a PCR product of 524 bp in size (Figure 2A).

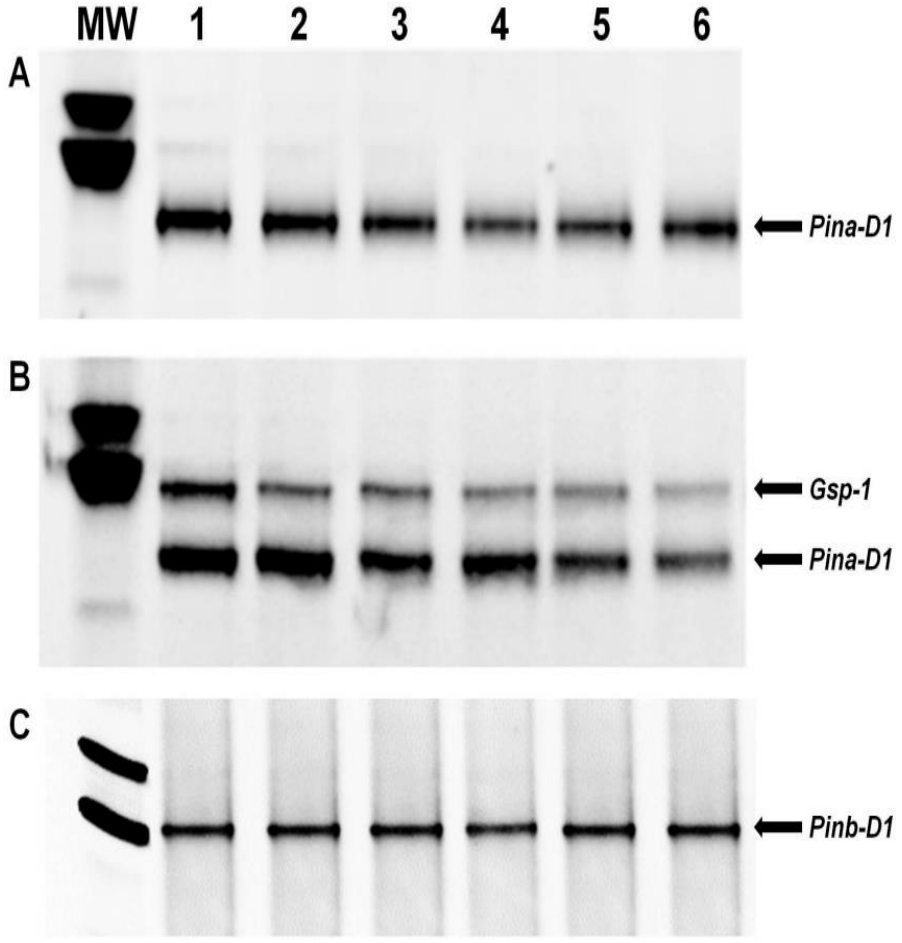

Figure 2. (**A**) *Pina-D1* amplification with primer LIL1; (**B**) *Pina-D1* amplification with primer MAS;(**C**) *Pinb-D1* amplification with primer LIL2. Lanes represent as follows: (**1**) cv. Chinese Spring *(Pina-D1a/Pinb-D1a*); (**2**) CWI 67429; (**3**) CWI 57816; (**4**) CWI 67483; (**5**) CWI 73046; and (**6**) CWI 72689.

When the primer MAS was used, the results for *Pina-D1* amplification presented two amplicons (Figure 2B), one of them with 516 bp (*Pina-D1*) and the other with 564 bp that corresponded to the grain softness protein (*Gsp-1* gene). All accessions showed the expected amplification product of 597 bp for the *Pinb-D1* gene (Figure 2C). Therefore, no apparent polymorphism was detected in any of the accessions, and the amplicons seemed similar to the ones of cv. Chinese Spring (*Pina-D1a*, *Pinb-D1a*).

Enzymatic Digestion and Sequencing of Pinb-D1 Alleles

Several specific restriction enzymes were used to digest the amplicons of *Pina-D1* and *Pinb-D1* in order to screen the Iranian landraces that were tested for the most common mutations for *Pina-D1* and *Pinb-D1*. The *Pina-D1* amplicons were digested with *Msp*I (used to detect the *Pina-D1m* allele), but no accession provided a positive result for this allele (Figure 3). Additionally, the *Pina-D1* amplicons were also digested with *Dde*I (used by Guzmán *et al.* (2012) to detect polymorphism in *Pina-D1*), but again no polymorphism was detected (data not shown).

However, several *Pinb-D1* alleles were found in our accessions using specific restriction enzymes (Figure 4). We found 11 accessions with the *Pinb-D1b* allele (positive for *Bsr*BI digestion), 80 accessions with *Pinb-d1p* (negative for *Pfl*MI digestion), and 175 accessions with the *Pinb-D1ab* allele (negative for *Apa*I digestion) (Table 1). The alleles *Pinb-D1c* and *Pinb-D1e* (digestions with *Pvu*II and *Bst*XI, respectively)

were not found in any of our accessions (data not shown).

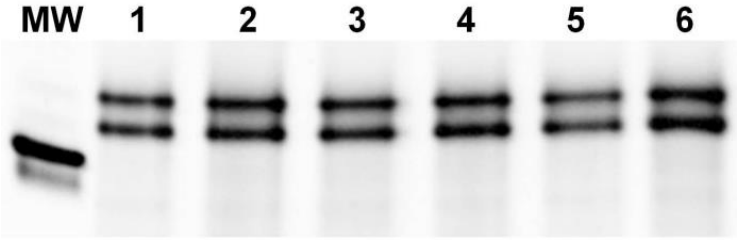

Figure 3. *Pina-D1* digestion with *MspI* endonuclease. Lanes represent as follows: (**1**) cv. Chinese Spring (Pina-D1a/Pinb-D1a); (**2**) CWI 67429; (**3**) CWI 57816; (**4**) CWI 67483; (**5**) CWI 73046; and (**6**) CWI 72689.

To detect more variability in the *Pinb-D1* gene, the remaining five accessions that did not show any differences when compared with those of cv. Chinese Spring in the above-mentioned analysis were selected for the sequencing of their *Pinb-D1* gene. The sequences obtained were compared with the sequence of the *Pinb-D1a* allele of cv. Chinese Spring (NCBI ID: DQ363913). All these accessions showed a G/A transition, resulting in a codon change at position 419 (TGC → TAG). According to a BLAST analysis performed and the literature reviewed, this mutation was not reported previously, and therefore we consider this allele to be novel. Following the order of the Wheat Gene Catalogue, we tentatively proposed to name this allele *Pinb-D1ak*. In addition, the results obtained with the restriction enzymes were corroborated by sequencing the *Pinb-D1* gene in few of the accessions that resulted in a positive for other alleles (*Pinb-D1p* and *Pinb-D1ab*). As all the accessions used in the study showed a mutation in *Pinb-D1* gene that explained their hard grain texture, it was assumed that they carried the *Pina-D1a* allele for *Pina-D1* gene.

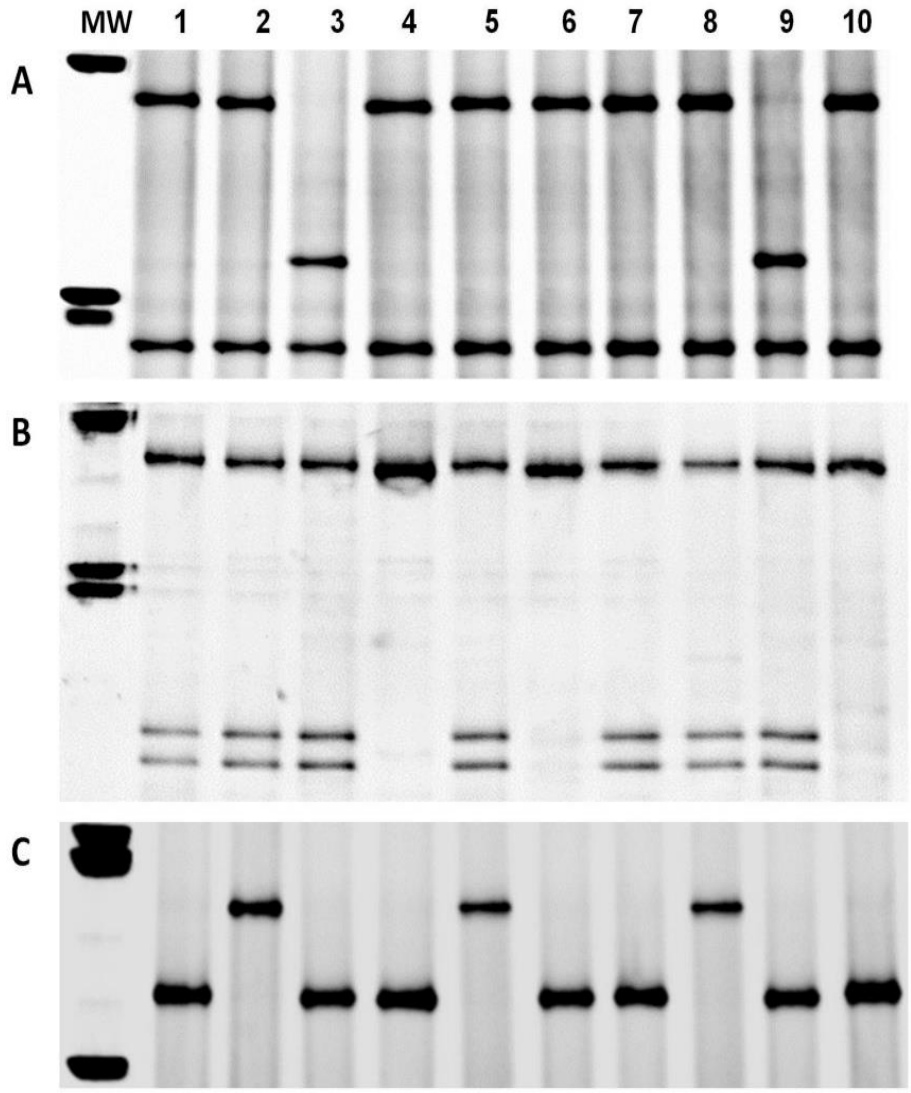

Figure 4. *(**A**) Pinb-D1 digestion with BsrBI endonuclease; (**B**) Pinb-D1 digestion with PflMI endonuclease; (**C**) Pinb-D1 digestion with ApaI endonuclease. Lanes represent as follows: (**1**) cv. Chinese Spring (Pinb-D1a); (**2**) CWI 67429 (Pinb-D1ab); (**3**) CWI 57816 (Pinb-D1b); (**4**) CWI 67483 (Pinb-D1p); (**5**) CWI73046 (Pinb-D1ab); (**6**) CWI 72689 (Pinb-D1p); (**7**) CWI 72663 (Pinb-D1a); (**8**) CWI 73075 (Pinb-D1ab);(**9**) CWI 66889 (Pinb-D1b); and (**10**) CWI 66921(Pinb-D1p).*

Table 1. List of *Pinb-D1* alleles that were searched for specific restriction enzymes in our study.

Allele	Mutation	N	Reference
Pinb-D1b	Gly/Ser at position 46	11	(Giroux and Morris, 1997; Wang *et al.*, 2008)
Pinb-D1ab	Gln/Stop at position 382	175	(Tanaka *et al.*, 2008); Wang *et al.*, 2008)
Pinb-D1p	Lsy/Asn at position 210	80	(Li *et al.*, 2008; Xia *et al.*, 2005)
Pinb-D1e	Trp/Stop at position 39	0	(Wang *et al.*, 2008; Morris *et al*., 2001)
Pinb-D1c	Leu/Pro at position 60	0	(Lillemo and Morris, 2000)

Amino Acid Sequence Analysis and Relationship with Grain Hardness

Both PIN proteins are synthesized as precursors or preproteins and contain four domains: a signal-peptide, N-terminal domain, mature protein, and C-terminal domain (Gautier *et al.*, 1994). Another distinctive feature of these proteins is the presence of a highly conservative cysteine backbone made up of ten cysteines and a tryptophan-rich domain. In the novel allele identified (*Pinb-D1ak*), which causes an amino acid change from cysteine to tyrosine at position 140 of the amino acid sequence, the cysteine change coincided with the tenth cysteine of the cysteine backbone of the PINB protein (Figure 5). In addition to this, the PROVEAN score obtained for the *Pinb-D1ak* allele (-4.2) indicated that this mutation likely has a strong effect on the protein function.

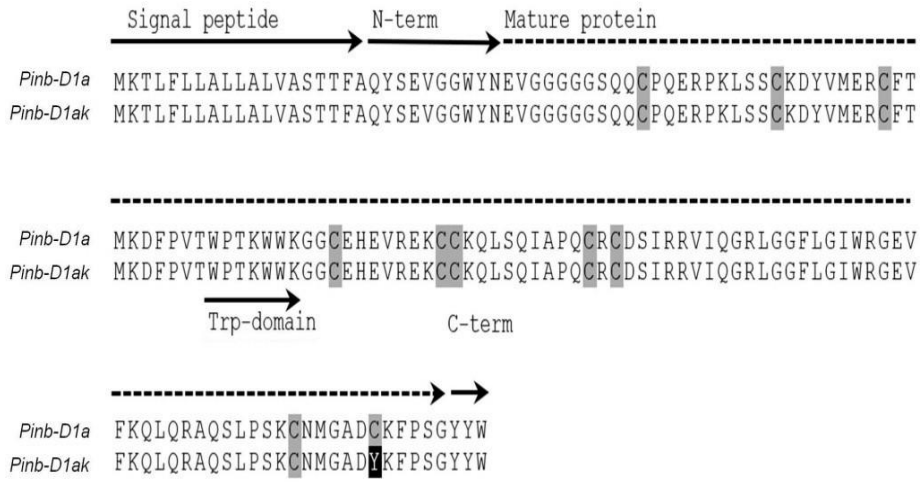

Figure 5. PINB-D1 protein structure and Trp-domain. The ten cysteine residues are marked in grey. Amino acid change C140Y for *Pinb-D1ak* allele is indicated in black.

The relationship between the grain hardness of the accessions with the new *Pinb-D1ak* allele and the rest of the accessions identified with the *Pinb-D1b*, *Pinb-D1ab*, and *Pinb-D1p* alleles was studied by analyzing the mean PSI of the group of accessions that had each allele. It was observed that the accessions with the *Pinb-D1ak* allele had a mean PSI value of 44.2 ± 2.93, slightly lower than that of the rest of the accessions groups with the *Pinb-D1b*, *Pinb-D1ab*, and *Pinb-D1p* alleles with mean PSI values of 45.91 ± 3.58, 46.91 ± 2.77, and 47.11 ± 2.64, respectively (Table 2). Therefore, it was observed that the accessions with the *Pinb-D1ak* allele had harder grains than those of the accessions with other mutations, as lower values of the PSI (%) correspond to harder grains (Gautier *et al.*, 1994).

Table 2. Allelic composition for puroindoline genes and the PSI values of the lines evaluated.

Puroindoline Composition	**N**	**PSI (% ± s.d.)**	**Range**
Pina-D1a/Pinb-D1b	11	45.91 ± 3.58 ^{AB}*	40–50
Pina-D1a/Pinb-D1ab	175	47.13 ± 2.70 ^{A}	40–50
Pina-D1a/Pinb-D1p	80	47.48 ± 2.64 ^{A}	41–50
Pina-D1a/Pinb-D1ak	5	44.20 ± 2.93 ^{B}	41–49

s.d.: Standard deviation; * letters indicate significantly ($p < 0.05$) different means groups.

Discussion

Landraces and old cultivars possess variability for different genes that may not be present in modern cultivars, and that knowledge could be useful for modern breeding pro- grams to develop materials with novel differential properties. Because of this characteristic, efforts are being made to identify useful novel variations among the vast wheat genetic resources stored in germplasm banks. In this regard, the CIMMYT's wheat germplasm bank holds a huge collection (more than 100,000 accessions) that is being characterized by different means (Lillemo and Morris, 2000; Vikram *et al.*, 2016) in order to possibly use its unexploited variation for the genetic improvement of the breeding program. Within the CIMMYT's collection, there is a large set (6800 accessions) of landraces with Iranian origin since Iran is one of the centers of diversity for the common wheat. This set of materials has shown great genetic diversity (Vikram *et al.*, 2021), including interesting

variations for grain quality traits related to both end-use and nutritional properties (Maryami *et al.*, 2020).

In this study, the grain texture of a set of Iranian bread wheat landraces was analyzed and characterized at the molecular level for puroindoline composition. Since grain hardness is one of the most important wheat quality traits, its genetic control has been extensively studied, and a lot of alleles of the two puroindoline genes involved (*Pina-D1* and *Pinb-D1*) have been identified. The *Pina-D1a* and *Pinb-D1a* alleles are associated with a soft grain texture (Giroux and Morris, 1997), while most of the other described alleles of *Pina-D1* and *Pinb-D1* are associated with a hard grain texture. The variability of these genes was studied before in germplasm collections of diverse origins. For example, Cane *et al.* (2004) examined the polymorphism of these genes in Australian wheat cultivars; Ayala *et al*. (2013) screened Mexican landraces, finding three different *Pin-D1* alleles leading to hard grain; Ma *et al.* (2017) characterized a large collection, mainly from China but with some cultivars from other countries (USA, Europe, Japan, etc.), to find six and nine different *Pina-D1* and *Pinb-D1* alleles, respectively. However, Iranian materials have been not studied in detail for the variability of these important genes. In our study, all the accessions were previously evaluated for grain hardness and had either hard or semi-hard grains. This indicated that the Iranian landraces selected for the current study likely carry mutations in the puroindoline genes that lead to a loss of function in their respective puroindoline proteins. Therefore, the probability of detecting mutations for the *Pina-D1* or *Pinb-D1* genes was

rather high.

First, using specific restriction enzymes, we searched different alleles that were previously described to cause changes in the PINA or PINB protein sequence or their absence and lead to hard endosperm. The *Pinb-D1b* allele was found, which consists of a single nucleotide change at position 46 in the *Pinb-D1* sequence that confers a glycine to serine change in the expressed protein. This change in the sequence resides near the tryptophan-rich domain and probably changes the secondary or tertiary structure of the protein, leading to hard endosperm (Giroux and Morris, 1997). The *Pinb-D1b* allele was detected in 11 accessions, which corresponded to a frequency of 4% of the total of our samples. This allele was found in different studies in landraces from different countries such as Spain (Ayala *et al.,* 2016), Mexico (Ayala *et al.,* 2013) and China (Chen *et al.,* 2007; Li *et al.,* 2008) with a frequency similar to that of our study. However, in other collections such as in a study of landraces from Poland (Przyborowski *et al.,* 2020), *Pinb-D1b* had a frequency of 21% of the total, which was much higher than the frequency of our study. It was also very frequent in cultivars from Norway, Sweden, and Finland (Lillemo and Morris, 2000). Therefore, we deduced that *Pinb-d1b* is widely distributed in America, Asia, and Europe, although in Europe the frequencies of *Pinb-D1b* in landraces are higher than in other regions.

Another allele found in our materials was *Pinb-d1ab,* which has a transition (C/T) at the 382 position, leading to the appearance of an early stop codon in the deduced mature protein (Gln→Stop) (Tanaka *et al.*, 2008; AACC, 2000). The presence of this premature stop codon causes

the synthesis of a non-functional PINB protein, which explains the hard grain in genotypes with this mutation. This mutation is very similar to the ones present in *Pinb-D1f*, *Pinb-D1e*, and *Pinb-D1g* alleles (Morris *et al.*, 2001; Przyborowski *et al.*, 2020). These mutations lead to an increase in grain hardness by overriding the correct expression of the PINB protein through the appearance of premature stop codons in other regions of the gene. The *Pinb-d1ab* allele was detected in 175 accessions, equivalent to a frequency of 64.6% of our total collection. The *Pinb-d1ab* allele was first discovered in two accessions from Afghanistan (Tanaka *et al.*, 2008). Later, it was detected in landraces from China (in 19.1% of the landraces studied) (Wang *et al.*, 2008) and from Pakistan (in 1% of the genotypes) (Khurshid and Ahmad, 2021). Therefore, it seems that both the origin and distribution of the *Pinb-d1ab* allele is exclusive to the Asian continent.

The *Pinb-D1p* allele was also found in our collection; this allele consists of an adenine deletion at the 210 position, resulting in a lysine to asparagine change and then a premature stop codon in the mature protein (Xia *et al.*, 2005). The *Pinb-D1u* (Chen *et al.*, 2007) and *Pina-D1l* (Pan *et al.*, 2004) alleles have similar mutations, characterized by the deletion of a nucleotide in their sequence. The *Pinb-D1p* allele was found in 80 accessions, resulting in a frequency of 29.5% of the total of our collection. This frequency is lower than in the study of wheat landraces from Shandong (China), in which the frequency of the *Pinb-D1p* allele was 59.6% (Li *et al.*, 2008). However, in other screenings with accessions from Pakistan and Afghanistan, this allele showed low

frequency (Tanaka *et al.*, 2008). These studies indicate that this allele may have spread along the "Silk Road". Therefore, it appears that its distribution is only across the Asian continent, as in the case of *Pinb-d1ab*.

Finally, the sequencing of the *Pinb-D1* gene in the remaining accessions that did not show any of the mutations described above allowed for the detection of a novel allele (*Pinb-D1ak*) that had not been previously described. This allele caused a nucleotide change that generated a change from cysteine to tyrosine at the 140 position of the amino acid sequence. Several studies have shown that the conservation of domains in the PINA and PINB proteins is very important for the conservation of the soft texture of the grain (Corona *et al.*, 2001; Feiz *et al.*, 2009), for which the backbone of cysteines (whose function is the stabilization of the structure of the puroindolines) is essential (Douliez *et al.*, 2000). In this sense, it is understood that the *Pinb-D1ak* allele has a large effect on protein functionality according to the PROVEAN score obtained, because as previously mentioned, this allele causes a change in the tenth cysteine of the cysteine backbone. There are other alleles described with similar characteristics as they have changes affecting the cysteine backbone, such as *Pinb-D1x*, which also has an amino acid change (Cys57→Tyr) (Wang *et al.*, 2008); in general, amino acid changes affecting the cysteine backbone are not very frequent. Other alleles in which a single amino acid change in the protein leads to an increase in grain hardness are *Pinb-D1d* (Lillemo and Morris, 2000) and *Pinb-D1q* (Chen *et al.*, 2005). In these cases, both alleles have an amino

acid change within the tryptophan-rich domain: in *Pinb-D1d*, there is a change from tryptophan-44 to arginine, and in *Pinb-D1q*, there is a change from tryptophan-44 to leucine. All these alleles are particularly interesting since they encode a protein that could have a different functionality, which may lead to intermediate grain hardness levels between the hard endosperm caused by the *Pin-D1* null alleles and the soft endosperm associated with the *Pin-D1a* alleles. In fact, several studies showed that genotypes with the *Pina-D1b* allele (null) are harder than genotypes with the *Pinb-D1b* allele (Gly46→Ser) (Giroux *et al.*, 2000; Martin *et al.*, 2001). In the case of the novel allele *Pinb-D1ak* found in this study, it was preliminary associated with a slightly harder grain texture than that of the other alleles reported; further studies with more balanced populations in terms of the frequency of each *Pin-D1* allele or with near isogenic lines are necessary to assess the level of grain hardness associated with this novel allele.

Conclusions

Grain hardness is of utmost importance for wheat processors, end-users, and those involved in wheat breeding and improvement. The materials used in this study showed remarkable variability for the puroindoline genes. One of the four mutations found in *Pinb-D1* had not been previously described, was considered novel, and was named *Pinb-D1ak*. This mutation is in the tenth cysteine of the protein backbone, which is a highly conserved region. On average, accessions with the *Pinb-D1ak* allele showed harder grains than accessions with other *Pinb-*

D1 alleles did. Thus, this new allele may be associated with harder kernels than other *Pin* alleles and could be used by breeding programs targeting different levels of kernel hardness. This study highlights the importance of conserving and characterizing wheat genetic resources that could be used as sources of genetic variability in breeding programs.

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CAPÍTULO III

Genetic variability for grain components related to nutritional quality in spelt and common wheat.

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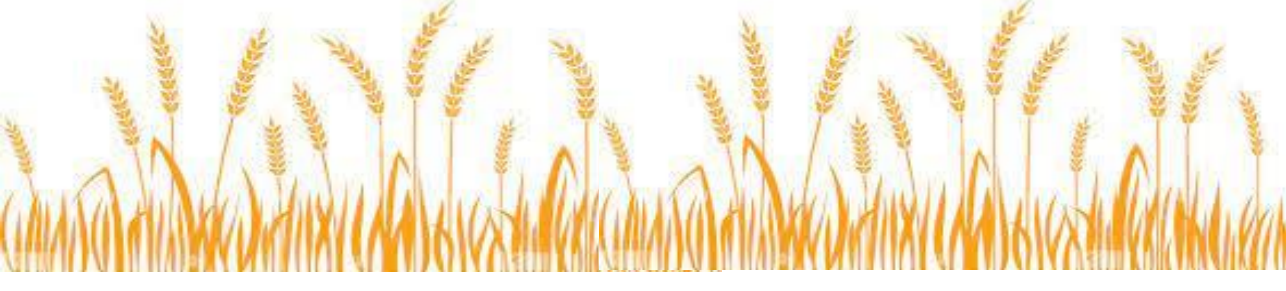

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Genetic Variability for Grain Components Related to Nutritional Quality in Spelt and Common Wheat

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ABSTRACT: Spelt (*Triticum aestivum ssp. spelta*) is part of the so-called ancient wheats. These types of wheats are experiencing a revival as they have been proposed to be healthier than conventional wheat. However, the given healthier condition of spelt is not substantiated by solid scientific evidence. The objective of this study was to analyze the genetic variability for several grain components, related to nutritional quality (arabinoxylans, micronutrients, phytic acid) in a set of spelt and common wheat genotypes to determinate if spelt is potentially healthier than common wheat. The results obtained indicated that within the compared species, there is a significant variation in the nutritional compounds, and it is not truthful and accurate to state that one species is healthier than the other. Within both groups, genotypes showing outstanding values for some traits were detected, which could be used in breeding programs to develop new wheat cultivars with good agronomic performance and nutritional quality.

KEYWORDS: *ancient wheats, nutritional quality, fiber, phytic acid, micronutrients*

Resumen

La espelta (*Triticum aestivum* ssp. *spelta*) forma parte de los llamados "trigos antiguos". Este tipo de trigos está experimentando un renacimiento, ya que se ha propuesto que son más saludables que el trigo convencional. Sin embargo, la supuesta condición más saludable de la espelta no está corroborada por pruebas científicas sólidas. El objetivo de este estudio fue analizar la variabilidad genética para varios componentes del grano relacionados con la calidad nutricional (arabinoxilanos, micronutrientes, ácido fítico) en un conjunto de genotipos de espelta y trigo harinero para determinar si la espelta es potencialmente más saludable que el trigo harinero. Los resultados obtenidos indicaron que dentro de las especies comparadas existe una variación significativa en los compuestos nutricionales, y no es veraz y exacto afirmar que una especie es más saludable que la otra. Dentro de ambos grupos se detectaron genotipos con valores sobresalientes en algunos caracteres, que podrían utilizarse en programas de mejora genética para desarrollar nuevos cultivares de trigo con buen rendimiento agronómico y calidad nutricional.

Palabras clave: Trigos antiguos; Calidad nutricional; Fibra; Ácido fítico; Micronutrientes

Abstract

Spelt (*Triticum aestivum* ssp. *spelta*) is part of the so called «ancient wheats». These types of wheats are experiencing a revival as they have been proposed to be healthier than conventional wheat. However, the given healthier condition of spelt is not substantiated by solid scientific evidence. The objective of this study was to analyze the genetic variability for several grain components, related to nutritional quality (arabinoxylans, micronutrients, phytic acid) in a set of spelt and common wheat genotypes to determinate if spelt is potentially healthier than common wheat. The results obtained indicated that within the compared species, there is a significant variation in the nutritional compounds, and it is not truthful and accurate to state that one species is healthier than the other. Within both groups genotypes showing outstanding values for some traits were detected, which could be used in breeding programs to develop new wheat cultivars with good agronomic performance and nutritional quality.

Keywords: Ancient wheats; Nutritional quality; Fiber; Phytic acid; Micronutrients

Introduction

Wheat is the world's most widely grown crop, occupying 221 million hectares and accounting for a quarter of total cereal production, with 771 million tons produced in 2021 (FAOSTAT, 2021). It is the staple food for about 40% of the world's population providing between 20% and 50% of total caloric intake in temperate countries. However, wheat is more than a source of calories as its grain contains significant amounts of other nutrients essential for a correct physical and mental development and a healthy life (Shewry, 2009). In fact, scientific evidence shows that regular consumption of wheat-based foods, preferably whole grains, provides health benefits such as reduced risks of obesity or overweight, type 2 diabetes, blood pressure and some cancers (Jones *et al.*, 2020).

The wheat grain is rich in protein, showing around 12-14% in average. Because of its widespread consumption, it accounts for almost 20% of total global dietary protein. In addition to its nutritional importance, a large part of the wheat grain protein is composed by the gluten proteins, which are responsible of the unique properties of the wheat dough that allow the preparation of hundreds of different foods appealing for consumers. Because of this, grain protein content (GPC) is of great interest in wheat genetic improvement programs, which generally aim to increase it, although that is difficult due to the negative relationship of this trait with grain yield. Furthermore, several micronutrients important for human nutrition such as iron (Fe) and zinc (Zn) are also present in wheat grain in significant amounts, contributing

44% and 25% of the daily intake in developed countries, respectively (Shewry, 2009). This could be probably higher in the developing countries where wheat is the main staple food. In these last regions, more than 2 billion people suffer from certain degree of micronutrient deficiency (mainly Fe, Zn, or vitamin A) (Bouis *et al.*, 2011) which have made that breeding programs include these traits in their breeding pipelines to alleviate this problem (Govindan *et al.*, 2022). Related to this, phytic acid (PA) is another important molecule that acts as a primary phosphate reservoir in the seeds. However, due to its ability to chelate micronutrients such as Fe and Zn it is considered often as an antinutrient and thus, ideally, the new wheat cultivars developed to fight against malnutrition should have reduced PA content in the grain. However, PA has also been associated to the prevention of major health risks such as the cardiovascular diseases or cancer (Fardet, 2010).

Dietary fiber is other important component of wheat grain, being wheat products one of the main sources of this bioactive component, accounting approximately 40% of dietary fiber intake in UK (Pot *et al.*, 2012). The main types of dietary fibers in wheat grain are β-glucans and arabinoxylans (AXs), being the later by far the most abundant (Gebruers *et al.*, 2008). AXs are usually divided into two classes, depending on whether they are water extractable (WE-AXs) or non-extractable (WU-AXs). Both types have different effects on human health (Shewry and Hey, 2015) and on processing and end-use quality (Garófalo *et al.*, 2011). As in the case of micronutrients, breeding programs are starting to target these grain components as well, in order to develop novel wheat

cultivars with enhanced health properties (Tremmel-Bede *et al.,* 2020; Ibba *et al.*, 2021).

Among the wheat species currently grown, bread or common wheat (*Triticum aestivum* ssp. *aestivum*) is by far the most important species, covering around 95% of the total wheat cultivated area. However, the need for more sustainable agriculture and crop diversification have led to a renewed interest in other wheat species such as spelt wheat (*T. aestivum* ssp. *spelta*) (Alvarez, 2021). Spelt wheat is hulled wheat that forms part of the so called «ancient wheats». These types of wheats were important in the past but were replaced by modern wheat cultivars due to their reduced agronomic performance. Probably, the most important reason for the revival of this species is that it has been proposed to be a better source of bioactive components than conventional and hence suitable for producing healthier and more 'natural' food products. Although a number of studies on spelt grain composition have revealed significant variation (Ranhotra *et al.*, 1996; Gomez-Becerra *et al.,* 2010) the given healthier condition of spelt compared to modern wheat is not substantiated by solid scientific evidence. There are a limited number of systematic studies on the detailed composition of spelt versus currently grown common wheat cultivars and it would be useful to know more about spelt diversity for nutritional grain components and how it differs from common wheat. (Shewry and Hey, 2015; Longin *et al.*, 2016).

In addition to the interest in spelt as a crop, useful genetic variation found in this species could also be used as a source for the development

of more nutritious common wheat (Guzmán *et al.*,. 2014).

The objective of this study was to analyze the genetic variability for several grain components related to nutritional quality in a set of spelt and modern common wheat genotypes to determinate if: 1- spelt has a better grain composition from the nutrition and health point of view than modern common wheat and; 2- to identify superior genotypes that could be used in breeding programs to develop high yielding adapted novel cultivars with high nutritional quality.

Materials and methods

Plant material and field trials

For this study, 89 Spanish spelt wheat accessions and ten modern common wheat cultivars were used (Table S1). These 89 spelt accessions were originally provided by the National Small Grains Collection (USDA, USA) and Centro de Recursos Fitogeneticos (INIA, Spain) and were purified and analyzed in previous studies showing significant variability for different traits (Caballero *et al.,* 2004; Guzmán *et al.,* 2010).

These accessions are traditional landraces and have not been hybridized with modern wheat. Of the ten modern cultivars, nine of them were commercial Spanish common wheat cultivars commonly grown in Andalusia (South of Spain) and that represent well the diversity found in farmers fields. The other cultivar was Anna Maria, a modern spelt wheat cultivar released in 2018 and derived of hybridization of spelt with

modern common wheat. These 99 wheat genotypes were planted in 0.13 m^2 plots with two replicates in a randomized complete block design under full drip irrigation during 2019-2020 and 2020-2021 crop seasons in Cordoba (Andalusia, Spain). Weed, diseases, and insects were all well controlled. Nitrogen fertilizer was applied (pre-planting) at a rate of 50 Kg of N/ha and at tillering 150 additional units of N and enough amount of micronutrients (including Fe and Zn) were applied.

Grain quality traits

Thousand kernel weight (TKW, g) and test weight (TW, Kg/Hl) were obtained using the SeedCount SC5000 digital imaging system (Next Instruments, Australia). GPC (%) was determined by near infrared spectroscopy (NIR Systems 6500, Foss Denmark) calibrated based on AACC official methods 39-10.01, 55-30.01 and 46-11.02, respectively (AACC, 2010).

An energy dispersive X-ray fluorescence spectrometry instrument (EDXRF, Oxford Instruments, Abingdon, UK) was used to determine Fe (mg/Kg) and Zn content (mg/Kg) in grain. A Megazyme scale-down protocol was used to determine the concentration of PA in whole-meal flour (Hernández-Espinosa *et al.*, 2020), obtained with an Udy Cyclone type mill. The molar ratios of phytic acid:iron (PA:Fe) and phytic acid:zinc (PA:Zn) were also calculated. WE-AXS and total AXS (TOT-AX) were determined in both whole-meal and refined flour (obtained by milling in a Brabender Quadrumat Senior Mill) using the colorimetric method reported by Hernández-Espinosa *et al.* (2020). Because of the

grain amount needed, the field repetitions were mixed to produce the refined flour. So, one unique data per genotype and year was obtained for TOT-AX and WE-AX in refined flour. The amount of mixed-linkage β-glucans in whole-meal flour samples was determined using a Megazyme kit (Megazyme, Bray, Ireland) according to AACC 32-23.01 standard method (AACC., 2010). β-glucans content was only determined in 14 of the genotypes (eleven spelt accessions and three modern wheat cultivars) of the study due to lack of enough grain in the rest of the samples to perform the analysis. Duplicate analyses were carried out on each sample.

Statistical methods

A multivariate analysis with all quality traits measured was performed by a principal component analysis using the covariance matrix between all genotypes. The comparison between both species sets was carried out for each trait analyzed by the *t*-Student test.

For spelt set, data were analyzed by an analysis of variance (ANOVA) using genotype, year and genotype × year as variation sources. The means were compared by the Tukey method. The differences among the value of each genotype and the mean of the common wheat genotype using as control were used to value the potential of the spelt genotypes for wheat breeding.

Correlation analysis between the measured traits was performed and represented in a matrix indicating significance values. Statistical analyses were carried out using Rstudio (version 4.2.1, Vienna, Austria.)

and Infostat software (version 29-09-2020, Cordoba, Argentina).

Results

Spelt versus common wheat

Diverse quality traits were measured for all materials used in this study; these data are shown in Table S2. For all these traits, the spelt genotypes showed large ranges of variation across the two years of the study compared with the common wheat cultivars used as controls (Fig. 1). The variation between the two data sets (90 spelt genotypes and 9 modern common wheat cultivars) was analyzed by a Principal Component Analysis. Up to 76.4% of the observed variation was determined by the PC1 (56.4%) and PC2 (20.0%). These two new variables permitted easily to discriminate between the spelt and modern common wheat genotypes (Fig. 2). Furthermore, this analysis showed that cv. Anna Maria, modern spelt wheat, was closer to the modern cultivars group than to the spelt genotypes, indicating that, at least in terms of quality traits, it seemed more similar to modern common wheat cultivars than to the spelt genotypes.

Almost all traits showed significant differences between the two groups (Table 1); for some of them the differences were small, such as TW, WE-AXs or β-glucans, while for others such as Fe, Zn, PA and GPC were large. In terms of protein content, the spelt genotypes showed more than two points of percentage difference.

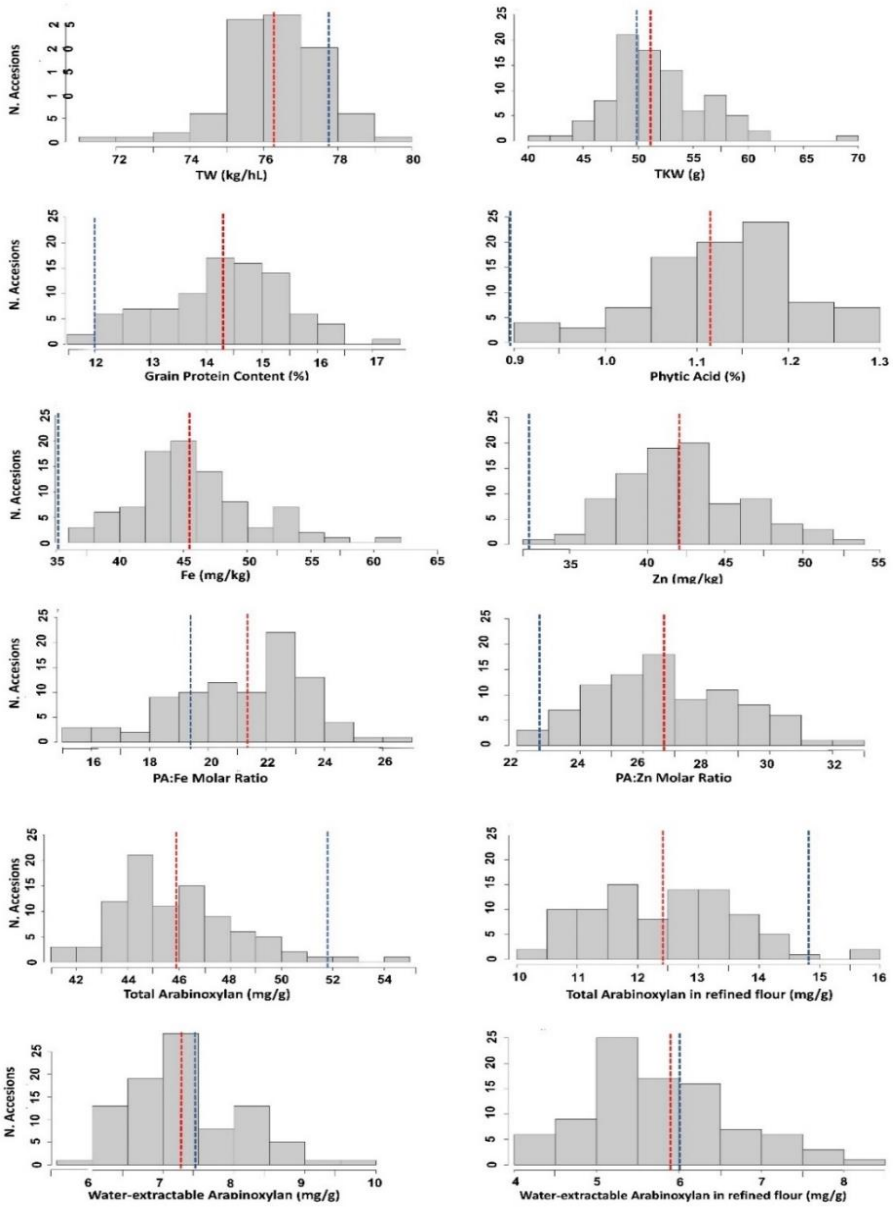

Figure 1. Number of spelt genotypes found in each range of variation for the different quality traits evaluated across the two cropping cycles of the study. Red and blue dots lines indicate the mean value of spelt genotypes and modern common wheat cultivars groups, respectively (averaging genotypes and years).

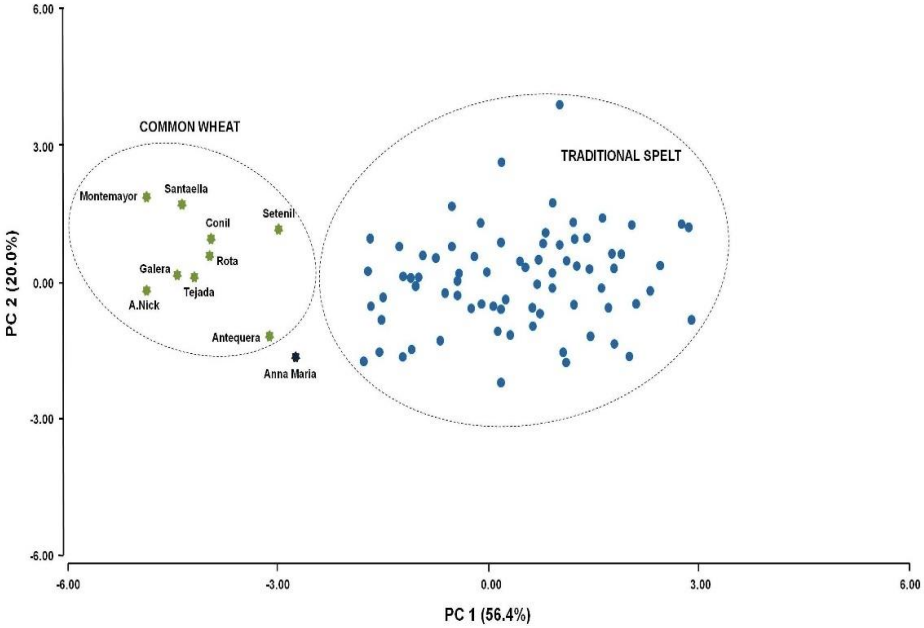

Figure 2. Principal component analysis of the quality traits analyzed. The distribution of the spelt landraces is shown in blue spots and that of the modern common wheat cultivars in red spots. Anna Maria, a modern spelt wheat cultivar, is shown in a green spot.

Similarly for micronutrients content, the Fe and Zn grain content was in average 10 mg/Kg higher in the spelt genotypes than in the common wheat genotypes; the PA content was also around two-thirds higher in the spelt group, which led to have significant higher PA:Fe and PA:Zn too. For the traits related with dietary fiber, WE-AXs did not show significant differences between the two groups in both whole-meal and refined flour; TOT-AXs and β-glucans were higher in common modern wheat cultivars (in the case of TOT-AXs in both whole-meal and refined flour).

Table 1. Average values of the common wheat and spelt groups (averaging genotypes and years) and result of the t-test done between both values.

Trait	Common wheat (n=9) (mean ± s.d.)	Spelt wheat (n=90) (mean ± s.d.)	t-value
TW (Kg/hL)	77.60 ± 2.77	76.25 ± 1.84	3.98***
TKW (g)	49.73 ± 6.03	51.68 ± 4.82	-2.25*
GPC (%)	11.87 ± 1.14	14.28 ± 1.75	-8.09***
Fe (mg/Kg)	32.63 ± 3.74	45.63 ± 5.49	-13.89***
Zn (mg/Kg)	32.79 ± 5.31	42.31 ± 5.55	-9.85***
PA (g/100g)	0.73 ± 0.09	1.12 ± 0.11	-20.39***
PA:Fe	19.34 ± 3.66	21.16 ± 3.03	-3.38***
PA:Zn	22.49 ± 3.31	26.66 ± 3.34	-7.14***
TOT-AX (mg/g)	51.01 ± 3.29	45.90 ± 3.74	7.89***
WE-AX (mg/g)	7.56 ± 2.07	7.32 ± 1.97	0.69 ns
TOT-AX (Ref.) (mg/g)	15.32 ± 1.65	12.46 ± 1.95	5.99***
WE-AX (Ref.) (mg/g)	6.15 ± 0.93	5.80 ± 1.03	1.40 ns
β-glucan (mg/g)	7.31 ± 0.52	6.64 ± 0.70	2.59*

TW, test weight; TKW, thousand kernel weight; GPC, grain protein content; Fe, iron content; Zn, zinc content; PA, phytic acid; PA:Fe, phytic acid:iron molar ratio; PA:Zn, phytic acid:zinc molar ratio; TOT-AX, total arabinoxylan; WE-AX, water-extractable arabinoxylan; Ref., refined flour.
***, *: significant at 99.9 and 95%; ns: not significant.

Variation of grain quality parameters in spelt

ANOVA was carried out to identify what factors had a larger contribution to the variation found for the quality traits in the spelt set (Table 2). In all cases except for WE-AXs, the genotype was by far the most important factor, explaining a larger percentage of the variation.

Table 2. Effects of genotype, year and their interaction on quality traits in spelt accessions. Squares sum (Sq. sum), % of the total squares sum of squares from ANOVA analysis, and broad-sense heritability (H2) are indicated.

Trait	Genotype Sq. Sum (%)	Year Sq. Sum (%)	Genotype × Year Sq. Sum (%)	Error Sq. Sum (%)	H^2
TW	601.35*** (49.41)	143.39*** (11.78)	186.41ns (15.32)	285.85 (23.49)	0.31
TKW	6853.18*** (82.16)	500.42*** (6.00)	375.34ns (4.50)	612.27 (7.34)	0.73
GPC	466.72*** (42.58)	23.27*** (2.12)	156.49ns (14.28)	449.51 (41.01)	0.28
Fe	7470.74*** (69.15)	0.76ns (0.01)	1483.99** (13.74)	1.848.59 (17.11)	0.56
Zn	5810.53*** (52.54)	286.87*** (2.59)	1677.08ns (15.16)	3.284.89 (29.70)	0.37
PA	2.50*** (56.03)	0.22*** (4.88)	0.69ns (15.42)	1.06 (23.67)	0.39
PA-Fe	1857.58*** (56.50)	46.85** (1.43)	589.49ns (17.93)	793.76 (24.14)	0.38
PA-Zn	1785.84*** (44.91)	0.56ns (0.01)	806.49ns (20.28)	1.383.48 (34.79)	0.25
TOT-AX	2103.42*** (41.90)	709.81*** (14.14)	942.07ns (18.77)	1.264.41 (25.19)	0.20
WE-AX	218.60*** (15.61)	1059.45*** (75.65)	64.44*** (4.60)	58.03 (4.14)	0.06
β-glucan	19.10*** (69.87)	0.24ns (0.88)	3.25ns (11.89)	4.74 (17.35)	0.50

TW, test weight; TKW, thousand kernel weight; GPC, grain protein content; Fe, iron content; Zn, zinc content; PA, phytic acid; TOT-AX, total arabinoxylan; WE-AX, water-extractable arabinoxylan. ***, *: significant at 99.9 and 95%; ns: not significant.

On average, the genotype explained 51.1% of the variation of the different analyzed traits, with traits such as WE-AXs for which the genotype contribution was smaller (15.6 % of the variation) and others such as TKW and Fe content for which it was outstanding (82.1 and 69.1% of the variation, respectively).In the case of TOT-AX and WE-AX in refined flour, the statistical analysis of these traits showed significant differences between both harvest years. On the contrary, the differences among genotypes were only significant for WE-AX.

The β-glucans content was measured on 14 selected spelt genotypes, together with two cultivars of common wheat (cv. Setenil and Tejada). For this trait, the genotypes showed significant differences among them; but the effects of the year and genotype × year interaction were not significant.

Identification of superior spelt genotypes for its use in breeding

The use of traditional spelt wheat has two possible ways for cereal breeding. On the one hand, it could serve as a donor of specific traits for modern common wheat breeding, by crossing it with modern materials and following selection of the desirable traits. On the other hand it could be used as crop, using the traditional varieties directly in the field or to breed modern spelt cultivars more adapted to current agriculture conditions. Consequently, the traits measured in the current study should be valued according with the concrete breeding finality: introgression in common wheat or development of new spelt cultivars. In this respect, the

broad-sense heritability values showed in Table 2 are key for establishing the real possibility of successful transference of these traits to modern wheat by hybridization and posterior selection.

The mean value for each trait for each genotype (averaging years) and the difference in percentage of this mean value with the average value of the nine modern common wheat cultivars using as checks were calculated (Tables S3a and S3b). Based on this analysis it was shown that eleven spelt genotypes, including cv. Anna Maria, present TW values higher than the control mean. Nevertheless, this trait could be influenced by the oblong shape of the spelt grain, and consequently many spelt genotypes have low values. In any case, the relatively low broad-sense heritability (H^2) value (0.31) suggested that this trait has an important environmental component and its introgression into modern wheat could be difficult. By comparing the different spelt genotypes, several of them (ESP-80, ESP-281, ESP-384 and ESP-387) showed higher values than cv. Anna Maria.

In the case of grain size (TKW), the H^2 value was the highest (0.73), which suggested that this trait was more highly dependent on the genotype and could be transferred successfully to modern wheat. In this respect, up to 58 spelt genotypes had higher values than those of the common wheat controls (49.73 g), with four genotypes exhibiting at least 20% higher TKW values (accessions ESP-36, ESP-244, ESP-245 and ESP-272) than the controls. Among these four spelt lines, the accession ESP-36 had the highest TKW values (TKW = 68 g) which was however associated with a low TW value (73.2 Kg/Hl, -5.6% compared to the

modern common wheat controls). The ESP-244 genotype combined large grains (TKW = 60 g, 22% more than the modern common wheats) with an acceptable TW (76.6 Kg/Hl, -1.2 % compared to the modern common wheat controls). The spelt cv. Anna Maria presented low grain size (42.76 g), which opens the possibility to develop new modern spelt cultivars with better grain size by using some of the materials analyzed in this study.

The correlation analysis (Table S4) showed no negative association between TKW and the protein content (GPC), which suggests that the development of materials with large grain size and high protein content could be possible. In fact, several spelt accessions had greater GPC values compared to the average GPC found in common wheat (11.87%). From those, the spelt genotypes ESP-51, ESP-84 and ESP-249 were probably the most interesting, as they combined more than 25% of the GPC found in common wheat controls and also had good grain morphology characteristics (TW and TKW values similar or higher than that of common wheats), which indicated a good capacity of those genotypes to accumulate protein in large, not shriveled grains.

The previously analyzed traits mostly influence wheat technological quality which is different than nutritional quality, where the presence and amount of the different grain components establishes the differences between superior and inferior genotypes. A large part of the spelt accessions showed significantly greater concentration of micronutrients (Fe and Zn) than the common wheat controls (32.63 and 32.79 mg/Kg) or the spelt cultivar Anna Maria (36.86 and 34.04 mg/Kg),

with few of them showing outstanding values such as accessions ESP-245 and ESP-288 for Fe content (60 and 57 mg/Kg, respectively) or accessions ESP-94 and ESP-252 for Zn content (52 and 51 mg/Kg, respectively). The H^2 values of these traits were moderate (Table 2), being higher for Fe content. Many of these spelt accessions with high Fe and Zn content showed at the same time high phytic acid content (PA) with a range between 0.906 and 1.289 g/100g (Table S3a). This last grain component showed high values in cv. Anna Maria (1.038 g/100g) as well, having only ten spelt genotypes with lower PA values (Table S3a). Although the PA has been associated with certain health properties, (Fardet, 2010). In relation to the micronutrients, such as Fe or Zn it shows chelate forming ability thus reduce the bioavailability of these micronutrients. Consequently, the accessions with the highest interest for breeding purposes focused on biofortification will be those ones having high oligoelements content and moderate PA content. This could be estimated by the PA:Fe and PA:Zn molar ratios. These values (PA:Fe and PA:Zn) were, in general, high in spelt genotypes with an high-moderate environmental component according with their H^2 values (Table 2). Nevertheless, 17 spelt genotypes exhibited lower PA:Fe molar ratio than those of the modern common wheat cultivars, but only one genotype (ESP-51) also showed low PA:Zn molar ratio. It is important to remark that regarding micronutrients content and potential bioavailability, the modern spelt cultivar Anna Maria showed slightly higher Fe and Zn contents than the common wheats but combined with higher PA, which led to larger PA:micronutrients molar ratios.

In terms of fiber content, only a few spelt genotypes had higher values than that of the common wheat controls for total AXs in both whole-meal and refined flour, and the differences were smaller than 11% (Table S3a). There were more remarkable differences for WE-AXs, for which genotypes ESP-224, ESP-227 and ESP-380 had more than 15% of WE-AXs in whole-meal flour and more than 22% in refined flour than in common wheat, which makes them interesting sources of this trait. In any case, the H^2 values of these traits suggested that the effect of the environment is high and, consequently, their transfer to common wheat could be complicated. For β-glucans (for which less spelt and common wheat cultivars were analyzed - Table S3b) only the accession ESP-300 showed a significantly larger amount compared to common wheat, although the difference was not very large (10%) compared to common wheat.

Discussion

During the last years, changes in the agri-food perception have generated a greater interest for ancient wheats both as crop per se and as donors of useful traits for modern wheat. In the past, these ancient wheats were mainly neglected due to their lower yield, poor adaptation to the agricultural mechanization, and because they required a special dehulling treatment in the mill to separate the chaff from the grain. Currently, their major interest is more related with their use in food.

In this context, spelt, a ancient wheat species neglected during the 20th century, is experiencing a great revival nowadays and is being

offered to customers by traditional and gourmet bakeries and many large retailers in Western countries. Probably, an important part of this success is because spelt has been proposed to be a great source of bioactive components and hence suitable for producing food products with enhanced health benefits. However, there are a limited number of systematic studies on the detailed nutritional quality of spelt wheat compared to common wheat species (Shewry, 2015; Ranhotra *et al.*, 1996; Longin *et al.,* 2016). In general, according to Shewry and Hey (2015), these comparisons have the problem to have been performed with a limited number of spelt or common wheat cultivars. This could have certainly biased the results, masking the true value of the spelt materials in some cases or attributing them superiority in terms of nutritional quality in others. Certainly, the number of available accessions of spelt or other ancient wheats is smaller compared with common wheat, but not so limited as to exclude the possibility of variation within them. Consequently, the evaluation of larger collections is essential, and this has motivated the development of larger studies (Curzon *et al.,* 2021; Tóth *et al.,* 2022). In this study 90 spelt and 9 common wheat genotypes have been compared in terms of grain nutritional components and other quality traits.

The technological quality must be evaluated with caution when the analyzed materials are ancient or old wheats. Changes in baking techniques throughout the last century generated materials adapted to these techniques, far from traditional baking, and consequently, the evaluation of ancient wheat according to modern parameters could not

be a good strategy. These characteristics are mainly demanded by millers are physical and chemical characteristics such as TW, TKW or GPC. Consequently, regardless of the rheological properties, the new materials must present characteristics of interest to the milling industry as a previous step. In this respect, within the accessions evaluated here, some materials presented high values of TKW, an important trait positively related with grain and flour yield (Matsuo and Dexter, 1980). Other studies have shown more moderate TKW values for spelt, although this depends of the materials evaluated: 'pure' spelt (without common wheat introgression) or modern spelt derived from crosses with common wheat (Tóth *et al.,* 2022; Winzeler *et al.,* 1994; Ratajczak *et al.,* 2020; Kulathunga *et al.,* 2021). In general, these last ones present larger grain size than pure spelt. However, in this study, genotypes of pure spelt with TKW values larger than 60 g have been found which has not been detected in other studies (Yoshioka *et al.,* 2019). Particularly, the accession ESP-36 had an outstanding high TKW value (68 g), higher than any other value found for this trait in large studies screening thousands of wheat accessions (Vikram *et al.,* 2021), which make it interesting to be used in the genetic dissection for this trait or by breeding programs aiming to develop new cultivars with very high grain size. On the contrary, the TW values in the spelt genotypes were low in general. This was expected, as these genotypes were not adapted to the testing area as in previous studies (Curzon *et al.*, 2021) and TW reflects well the adaptation of a genotype to a particular environment. Anyway, it was possible to identify spelt genotypes (accessions ESP-92, ESP-250, and

ESP-295), combining large grains and TW values as high as the ones of the common wheat checks, which could be useful for wheat breeding programs aiming to develop modern spelt and common wheat cultivars with higher milling quality.

Another important quality trait analyzed that has great importance for the industrial and nutritional quality, was GPC, which is in general negatively influenced by grain yield. However, there are cultivars with the ability to combine high values for both traits appreciated by farmers and food industry. For this reason, the search of genotypes with high TKW and GPC values is interesting for the development of new wheat cultivars with potential high grain yield and protein content. To breed competitive high protein cultivars, the accession ESP-216 is probably the most interesting material identified in the current study (37.5 and 2.6 % higher GPC and TKW, respectively, than the checks). The accession ESP-94 had also an outstanding GPC (17.2%), but in this case it could be due to a concentration effect due to the smaller grain size and lower test weight.

Among the nutritional quality traits analyzed in this study, Fe and Zn have gained notable importance in wheat improvement recently. This is mainly because millions of people suffer from some degree of these micronutrients deficiency in developing countries, which is named as 'hidden hunger' (Bouis *et al.*, 2011). This problem is not unknown in developed countries where access to food is not always parallel to good nutrition. Consequently, the development of modern genotypes with higher concentration of micronutrients, mainly Fe and Zn, is important

for several wheat-growing and wheat-consuming areas. In general, spelt analysed here showed significantly higher micronutrients content compared to the common wheat checks (32 vs. 45 mg/Kg for Fe, and 32 vs. 42 mg/Kg for Zn, respectively), which agrees with previous studies (Ruibal-Mendieta *et al.,* 2005). In fact, some spelt genotypes have been successfully used to breed biofortifed cultivars, such as Zincol-16, a cultivar developed by CIMMYT-HarvestPlus and released in 2016 in Pakistan (Govindan *et al.,* 2022). This cultivar has a great impact in the area (3.5 million metric tons produced in 2021). In particular, some of the spelt genotypes had very good results for the content of these micronutrients, with values higher than 60 mg/Kg for Fe (ESP-245) or 51 mg/Kg for Zn (ESP-252) and showing good grain sizes at the same time (>52 g for TKW). Spelt genotypes showing high micronutrients content but poor grain characteristics are not very interesting because the high micronutrients content is probably due to a concentration effect associated to low grain yield (Curzon *et al.,* 2021). However, all spelt genotypes showed higher phytic acid content than the common wheat checks, which could reduce the bioavailability of Fe and Zn due to its chelate forming ability (Eagling *et al.,* 2014). The same trend was found by Longin *et al.*, (2023), higher phytic acid values in the spelt group lead to higher phytic acid:Fe or Zn molar ratios than in the common wheat checks in most cases, something in principle negative from the nutritional point of view. The negative impact of phytic acid could be modulated by cultural practices during food preparation: for example, the proofing and fermentation process during baking has been showed to

reduce the phytic acid content (Longin, *et al.,* 2023; García-Estepa *et al.,* 1999) therefore, in regions where that practise is applied, a high phytic acid content should have lower negative impact on Fe and Zn bioavailability. In addition to this, phytic acid has been proven to be a powerful antioxidant with beneficial effects in several diseases such as cancer, increased cholesterol level, and diabetes (Upadhyay *et al.,* 2022). This could recommend its consumption in areas where the supply of micronutrients is guaranteed by a complete and diverse diet. Because of this, some of these spelt materials may be useful to develop wheat with flour carrying more antioxidant compounds for such areas.

Another grain component that is associated with positive effects on health is dietary fiber. In the current study, arabinoxylans (AXs), contrary to the case of the micronutrients described above, were higher concentrated in the common wheat cultivars than in spelt genotypes. This agrees with the finding of Gebruers *et al.* (2008) and Hernández-Escareño *et al.* (2015). Nevertheless, the variability of these components was large in spelt accessions, and some superior genotypes were identified, such as ESP-242 (also highlighted before due to its high Zn content). This showed higher values than the common wheat controls in both whole-meal and white flour. This accession was rich in soluble AXs, which is particularly interesting as this fiber type is also related to processing and end-use quality resulting a positive effect (Courtin and Delcour,. 2002). The amount of soluble AXs in this accession is far from the best source for this trait described in the literature, cv. Yumai-34 (9 vs. 14 mg/g) (Gebruers *et al.,* 2008). Although the data showed low

heritability in the current study, in several trials carried out with common wheat (Martinant *et al.*, 1999; Dornez *et al.*, 2008; Shewry *et al.*, 2010), the fiber content has been shown as a character with strong genetic control and high heritability. Consequently, although further studies should be carried out, for the increase of AXs content, due to the complexity of this trait where different genetic regions are involved, it could be interesting to use materials with higher AXs content than cv. Yumai-34 inside the breeding programs (Tremmel-Bede *et al.*, 2020).

Conclusions

In summary, the current study suggested that, within the compared species (spelt and common wheat), there is a significant variation in the nutritional compounds, and it is not truthful and accurate to state that one species is healthier than the other. Within both groups there are promising genotypes for some traits, but not combining high values for all traits. Consequently, the consideration of one species, *sensu lato*, as a healthier or more nutritious for food uses, is not acceptable; however, it is true that within these species there are genotypes with outstanding values for particular nutritional traits that could be used as a source of variation in breeding programs.

The data obtained in the current study indicated that some spelt genotypes could be used for improving traits such as grain size and protein, Fe or Zn content. Ideally, these materials could be hybridized with common wheat genotypes of high TW and low PA content, together with high AXs content (for which the spelt group has not showed

superiority). These crosses could be used for two different objectives: development of new common wheat cultivars or, alternatively, new modern spelt cultivars with good agronomic performance and high nutritional quality.

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CAPÍTULO IV

Processing and bread making quality profile of Spanish spelt wheat.

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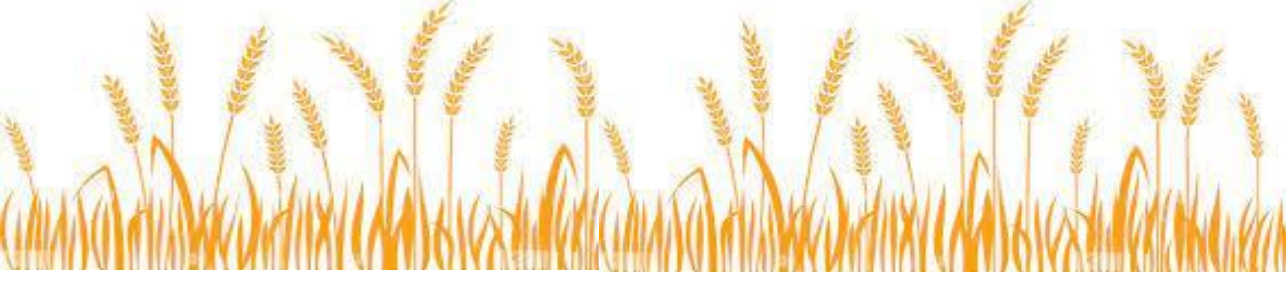

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Processing and Bread-Making Quality Profile of Spanish Spelt Wheat

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Abstract: Spelt wheat (*Triticum aestivum* L. ssp. *spelta* Thell.) is an ancient wheat that has been widely cultivated for hundreds of years. Recently, this species has been neglected in most of Europe; however, the desire for more natural and traditional foods has driven a revival of the crop. In the current study, eighty-eight traditional spelt genotypes from Spain, together with nine common wheat cultivars and one modern spelt (cv. Anna Maria) were grown during a period of two years in Andalucia (southern Spain). In each, several traits were measured in to evaluate their milling, processing, and end-use quality (bread-making). The comparison between species suggested that, in general, spelt and common wheat showed differences for most of the measured traits; on average, spelt genotypes had softer grains, higher protein content (14.3 vs. 11.9%) and gluten extensibility (alveograph P/L 0.5 vs. 1.8), and lower gluten strength (alveograph W 187 vs. 438 $\times 10^{-4}$ J). In the baking test, both species showed similar values. Nevertheless, the analysis of this set of spelt genotypes showed a wide range for all measured traits, with higher values than common wheat in some spelt genotypes for some traits. This opens up the possibility of using these materials in future breeding programs, to develop either new spelt or common wheat cultivars.

Keywords: wheat quality; genetic resources; ancient wheat; bread-making

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Resumen

El trigo espelta (*Triticum aestivum* L. ssp. *spelta* Thell.) es un trigo antiguo que se cultivó ampliamente en la Antigüedad. Esta especie se ha abandonado en la mayor parte de Europa; sin embargo, el deseo de alimentos más naturales y tradicionales ha impulsado un renacimiento del cultivo. En el presente estudio, ochenta y ocho genotipos de espelta tradicional de España, junto con nueve cultivares de trigo blando y una espelta moderna (cv. Anna Maria) se cultivaron durante dos años en Andalucía (sur de España). Se midieron varios rasgos en estos materiales para evaluar su calidad de molienda, procesado y uso final (panificación). La comparación entre especies sugiere que, en general, la espelta y el trigo blando mostraron diferencias en la mayoría de los rasgos medidos; en promedio, los genotipos de espelta tenían granos más blandos, mayor contenido proteico (14,3 frente a 11,9%) y extensibilidad del gluten (alveógrafo P/L 0,5 frente a 1,8), y menor fuerza del gluten (alveógrafo W 187 frente a 438 x 10^{-4} J). En la prueba de panificación, ambas especies mostraron valores similares. No obstante, el análisis de este conjunto de espeltas mostró un amplio rango para todos los rasgos medidos, con valores superiores a los del trigo blando en algunos genotipos para algunos rasgos. Esto abre la posibilidad de utilizar estos materiales en futuros programas de mejora genética, tanto para desarrollar nuevos cultivares de espelta como de trigo blando.

Palabras clave: Calidad del trigo; Recursos genéticos; Trigo antiguo; Panificación

Abstract

Spelt wheat (*Triticum aestivum* L. ssp. *spelta* Thell.) is ancient wheat that was widely cultivated in the Antiquity. This species has been neglected in most of Europe; however, the desire for more natural and traditional foods has driven to a revival of the crop. In the current study, eighty-eight traditional spelt genotypes from Spain, together with nine common wheat cultivars and one modern spelt (cv. Anna Maria) were grown during two years in Andalucia (South of Spain). Several traits were measured in these materials to evaluate their milling, processing, and end-use quality (bread-making). The comparison be-tween species suggests that in general spelt and common wheat showed differ-ences for most of the measured traits; in average spelt genotypes had softer grains, higher protein content (14.3 vs. 11.9%) and gluten extensibility (alveo-graph P/L 0.5 vs. 1.8), and lower gluten strength (alveograph W 187 vs. 438 x 10^{-4} J). For the baking test both species showed similar values. Nevertheless, the analysis of this set of spelt showed a wide range for all measured traits, with higher values than common wheat in some spelt genotypes for some traits. This opens the possibility of using these materials in future breeding programs, both to develop new spelt or common wheat cultivars.

Keywords: Wheat quality; Genetic resources; Ancient wheat; Bread-making

Introduction

Since the 1960s, the importance of plant genetic resources has gradually increased, as shown by the development of the "International Treaty on Plant Genetic Resources for Food and Agriculture" (https://www.fao.org/3/a-i0510e.pdf). The lack of genetic diversity in crops, globalization, and climate change, have shown how easy is for any pathogen or plague to quickly spread around the world (Gautam *et al.*, 2013). This could be a threat for food security, as modern agriculture is increasingly focused on few crops and few varieties within them (Esquinas-Alcazar, 2005) At the same time, the greater awareness of the need to use more sustainable and environmentally friendly agronomic techniques, together with the problems associated with Global Change, has boosted the search for alternative gene sources, which is one of the strategies used to develop more resilient cultivars under the conditions of global warming. In this context, ancient wheats and wheat wild wheat relatives, which have adapted to be grown in marginal zones under extreme conditions (Srivastava and Damania, 1989) are considered to host interesting and unexploited genetic variability that could be used in modern wheat breeding programs to develop more resilient cultivars. Among these ancient wheats, spelt (*Triticum aestivum* L. ssp. *spelta* Thell., 2n = 6× = 42, A^uA^uBBDD), originally obtained from the natural hybridization between emmer wheat (*T. turgidum* spp. *dicoccum* Schrank em. Thell., = 4× = 28, A^uA^uBB) and *Aegilops tauschii* ssp. *strangulata* Coss. (2n = 2× = 14, DD) in the Fertile Crescent (Near East), is the most cultivated species nowadays, and several spelt cultivars have

been bred for in order to improve their productivity (Campbell, 1997; Geisslitz, and Scherf, 2020). For this reason, some ancestral traits like the hulled grain or the semi-branching rachis, have been modified through crosses with common wheat (McFadden and Sears, 1945; Schmid and Winzeler, 1990; Winzeler, *et al.*, 1994; Suchowilska *et al.*, 2020).Consequently, two different types of spelt are now present in the farmers' fields: the traditional or pure spelt, and the modern spelt derived from hybridization with common wheat (Campbell, 1997). The variability of these two types of spelt is notably different, with the traditional spelt holding a greater genetic variability than modern spelt. Nevertheless, more studies comparing the variability of modern and traditional spelt are needed.

On the other hand, several studies suggested the exceptionality of the Iberian spelt (pol. *ibericum* Flaskb.) compared to the rest ofEuropean spelt (Bavarian group, pol. *bavaricum* Vav.), including the old studies of N.I. Vavilov (Salamini *et al.*, 2002; Dedkova, *et al.*, 2004; Elía *et al.*, 2004; Alvarez, 2021). So, while European spelt could derive from a secondary hybridization event between emmer wheat and hexaploid wheat, the Iberian spelt would be originated from the first hybridization event between emmer wheat and *Ae. tauschii* ssp. *strangulata* in Asia (Salamini *et al.*, 2002; Dedkova, *et al.*, 2004; Elía *et al.*, 2004; Alvarez, 2021). Furthermore, the spelt crop in Spain has been scarce until recent times and mainly based in traditional materials. The appearance of modern spelt in Spain is recent, and only two cultivars have been developed since 2018: cvs. Anna Maria and Viso. However, the

traditional Spanish spelt stored in Germplasm Banks is abundant (Alvarez, 2021). The current trend with this crop opens the opportunity of putting in value these old materials for their use in the current agricultural context, both as pure spelt or as a source of novel genetic diversity to develop modern spelt (Alvarez, 2021; Kulathunga *et al*., 2020) or common wheat cultivars.

Before this new trend on old crops, spelt has already been used in breeding programs as a source of resistance genes for some wheat diseases (Zeven *et al.,* 1968; McVey and Leonard, 1990; Zeller *et al.*, 1994; Dyck and Sykes, 1994; Singh *et al.*, 2006; Simon *et al.,* 2010; Mohler *et al.,* 2012; Singh *et al.,* 2013; Peng *et al.,* 2014; Dinkar *et al.,* 2020; Goriewa-Duba *et al.,* 2020). Now, in the context of the renewed interest in artisan and "more natural" food, spelt is used as raw material for the elaboration of food products (bread, biscuits, pasta, pancakes, etc.) which are present in many bakeries but also in large retailers. For this reason, studies on the processing quality of this crop have gained notable importance (Ranhotra *et al.,* 1995; Abdel-Aal *et al.,* 1997; Bojnanská and Francáková, 2002; Wilson *et al.,* 2008; Ratajczak *et al.,* 2020; Podolska *et al.,* 2020; Kulathunga *et al.,* 2021; Hadnađev *et al.,* 2022). Wheat processing quality is complex and varies depending on the wheat processor (millers or bakers) and based on the final products to be produced. Wheat millers for example, value grain size, test weight and texture, which are associated with the flour yield, and grain protein content, which is a partial indicator of wheat functionality (Matsuo and Dexter, 1980; Breseghello and Sorrells, 2006). Bakers on the other hand,

value the quantity and quality of the protein in flour, and the rheological properties of the dough made with it. For these reasons, the evaluation of new wheat materials must consider the quality requirements of all wheat processors including both millers and bakers.

Most of the studies conducted on the processing quality of ancient wheat, only included a limited number of accessions (Shewry, 2015) which impeded to have a clear understanding of the potential in terms of wheat processing, of such species. In general, the quality characteristics of these ancient wheats have been compared with modern wheat (common wheat – *T. aestivum* L. ssp. *aestivum*). Although this could be right, these data should be evaluated with caution. Any of these ancient wheats have been revived as a modern wheat substitute, and consequently to establish the quality characteristics of modern wheat as the reference could be clearly inadequate and leading to underestimate all ancient wheats. Obviously, spelt is not common wheat. The development of new cultivars of these ancient wheats should be complementary of the modern wheat in the context of the new agri-food industry.

The main objective of this study was the evaluation of a wide collection of Spanish traditional spelt accessions for some grain and technological quality traits, together with their comparison to one modern spelt (cv. Anna Maria) and several common wheat cultivars widely cultivated in Andalucia (South of Spain).

Materials and Methods

Plant material and field trials

Eighty-eight accessions of Spanish traditional spelt, together with ten modern wheat cultivars (nine common wheats and one modern spelt) were used (Table S1). These materials were planted in a randomized complete block design with two replicates during 2019-2020 and 2020-2021 crop seasons in Cordoba (Andalusia, Spain). Due to the high number of materials, the plot size was small (0.13 m^2) and, consequently, the grain yield was limited for some accessions that could be only evaluated for small-scale tests.

The 88 traditional spelt accessions were selected according to their grain proteins composition and origin from two wide collections originally provided by the National Small Grains Collection (USDA, USA) and Centro de Recursos Fitogeneticos (INIA, Spain) (Caballero *et al.*, 2001; Caballero *et al.*, 2004). Of the ten modern cultivars used as control, nine of them were commercial Spanish common wheat cultivars commonly grown in the South of Spain (cvs. Antequera, Arthur Nick, Conil, Galera, Montemayor, Rota, Santaella, Setenil and Tejada), which fall into different categories within the Spanish quality groups, depending on their performance on each environment. Cvs. Antequera, Conil, Galera, Rota and Tejada fall often within the Spanish quality group 1 (strong gluten wheat for mechanized bread-making), while cvs. Arthur Nick, Montemayor, Santaella, and Setenil produce grains that are usually classified as quality groups 2-3 (strong-medium gluten wheat for

semi-mechanized bread-making). The modern spelt (cv. Anna Maria) is a modern spelt cultivar obtained from hybridization between pure spelt and common wheat.

Grain and flour quality traits

Thousand kernel weight (TKW, g) and test weight (TW, Kg/Hl) were obtained using the SeedCount SC5000 digital imaging system (Next Instruments, Australia). The grain (GPC, %) and flour (FPC, %) protein content were determined by near infrared spectroscopy (NIR Systems 6500, Foss Denmark) based on AACC official methods 39-10.01 and 39-11.01, respectively, which were calibrated based on 46-11.02 method (AACC, 2010) Grain hardness was measured on samples of 100 kernels with the single-kernel characterization system (SKCS) (Perten Instruments, Springfield, IL, U.S.A.) (AACC, 2010). The polyphenol oxidase (PPO) activity was measured by absorbance at 475 nm according to Anderson and Morris (Anderson and Morris, 2001) and expressed in $Ug^{-1}min^{-1}$.

For the milling, the two field replicates of each genotype were mixed in order to obtain enough flour. The grain samples were processed applying AACC method 26-95 (AACC, 2010). All samples were milled into flour using a Brabender Quadrumat Senior mill (CW Brabender, Duisburg, Germany) and flour yield (%) was calculated. Measurement of sodium dodecyl sulfate (SDS) sedimentation volume (ml) was carried out according to Dick and Quick methodology (Dick and Quick, 1983) with the modifications introduced by Peña *et al.* 1990.

Alveographic and baking traits.

The dough tenacity (P), extensibility (L), tenacity/extensibility ratio (P/L), tenacity/swelling index ratio (P/G), elasticity index (Ie), and strength (W) were determined by AACC 54-30.02 method using a Chopin Alveograph (AACC, 2010). Due to the limited flour available, dough rheological properties were measured only on seven common wheat cultivars and 80 spelt accessions.

The bread-making process was conducted on four common wheat cultivars and 50 spelt accessions (only of these genotypes there was enough flour available to perform the test), using the direct dough method (AACC 10-10.03 method) and loaf volume (cc) was determined by rapeseed displacement using a volume meter (AACC, 2010).

Statistical methods

The comparison between both species sets was carried out for each trait analyzed by the t-Student test. A Pearson correlation analysis was carried out among the grain, flour and rheological traits within the Spanish spelt set.

For the spelt set, data were analyzed by an analysis of variance (ANOVA) using genotype, year and genotype × year as variation sources. Because cv. Anna Maria represented the current trend in spelt, their data for each measured traits were used as reference to evaluate and compare the values of each Spanish traditional spelt genotype. All statistical analyses were performed using Statistix software (version 9).

Results

Comparison among species

The data obtained for all materials evaluated (Tables S2 and S3) were grouped according to the species (common wheat vs. spelt) with the finality to compare the two groups. The mean values of each set were analyzed by the t-Student test (Table 1).

For grain or flour components, the differences between both species were in general small, but significant. The thousand kernel weight (TKW) of spelt was slightly higher than common wheat; however, spelt grains showed lower test weight (TW) values, probably due to the morphology of their grains that have, on average, an elongated shape. This had not influence on the flour yield, although the grain hardness, clearly lower in spelt, could have played a role on the flour yield obtained too.

The protein content was higher in spelt both in grain and in flour. But this has scarcely influence on the gluten strength measured by the SDS-sedimentation test, because the t-Student analysis indicated that the differences between both species are not significant (Table 1). On the contrary, there were highly significant differences for polyphenol oxidase (PPO) activity, for which the spelt group exhibited the double mean activity than the common wheat group.

Table 1. Average values of the common wheat and spelt groups (averaging genotypes and years) and result of the t-test done between both values.

Trait	No. genotypes (common : spelt)	Common wheat (mean ± s.d.)	Spelt (mean ± s.d.)	t-value
Grain/Flour components				
TW (Kg/hL)	9:89	77.60 ± 2.77	76.26 ± 1.84	3.94***
TKW (g)	9:89	49.73 ± 6.03	51.72 ± 4.81	-2.30*
GPC (%)	9:89	11.87 ± 1.14	14.27 ± 1.76	-8.01***
Hardness (%)	9:89	52.44 ± 15.07	15.78 ± 11.02	18.32***
Flour yield (%)	9:89	65.78 ± 6.06	67.53 ± 3.27	-2.78**
FPC (%)	9:89	10.22 ± 0.72	11.82 ± 0.98	-9.53***
SDS-sed (ml)	9:89	15.22 ± 2.65	15.86 ± 3.03	-1.22ns
PPO activity ($Ug^{-1}min^{-1}$)	9:89	4.25 ± 1.75	9.08 ± 2.06	-13.54***
Alveogram parameters				
P (mm)	7:80	141.57 ± 26.18	59.42 ± 16.48	16.93***
L (mm)	7:80	84.93 ± 18.13	123.83 ± 23.20	-6.10***
P/L (ratio)	7:80	1.79 ± 0.68	0.51 ± 0.24	15.27***
P/G (ratio)	7:80	7.08 ± 1.96	2.47 ± 0.89	16.33***
W (x 10^{-4} J)	7:80	437.71 ± 117.33	186.56 ± 57.04	14.16***
Ie (%)	7:80	61.33 ± 10.35	46.10 ± 6.45	8.00***
Loaf parameters				
Loaf Volume (cc)	4:50	778.00 ± 28.10	809.50 ± 57.64	-1.04ns

TW, test weight; TKW, thousand kernel weight; GPC, grain protein content; FPC, flour protein contents; SDS-sed, Sodium dodecyl sulfate sedimentation test; PPO activity, polyphenol oxidase activity; P, dough tenacity; L, dough extensibility; G: swelling index; W, dough strength; and Ie, elasticity index.
***, **, *: significant at 99.9, 99 and 95%; ns: not significant.

The alveograph parameters showed that while common wheat presented dough with high tenacity (P) and low/moderate extensibility (L), the spelt genotypes showed in general low to moderate tenacity (P) and high extensibility (L). In any case, the dough strength (W) was larger in common wheat than in spelt (Table 1). Nevertheless, within both sub-sets there were no significant differences in the bread-making quality of

the two groups (loaf volume), although the mean value of spelt was 30 cc higher than that of common wheat.

Variability for grain and flour quality traits in spelt

When the comparison was carried out among the spelt genotypes (Figure 1), both traditional and modern spelt (cv. Anna Maria), the data showed high variation among these genotypes for all measured traits in grain and flour (Table S2). The ANOVA analysis suggests high influence of the genotype in this variation (Table S4), although the differences between both years were also significant.

Most of the traditional spelt genotypes showed lower TW values than the cv. Anna Maria; however, the thousand kernel weight (TKW) of this last material was significantly lower than the values of the traditional spelt accessions (Figure 1).

The protein content, both in grain and flour, was highly variable; being cv. Anna Maria in the low part of the distribution in both cases (Table S2). This high protein content has scarce effect on the grain hardness, because in general the spelt genotypes showed soft or very soft grain; although some accessions showed values of semi-hard grain (Figure 1). The general lower grain hardness associated with the spelt accessions, was positively associated with flour yield.

The gluten strength measured as the SDS-sedimentation volume showed values among medium and high, with some exceptions (Figure 1).

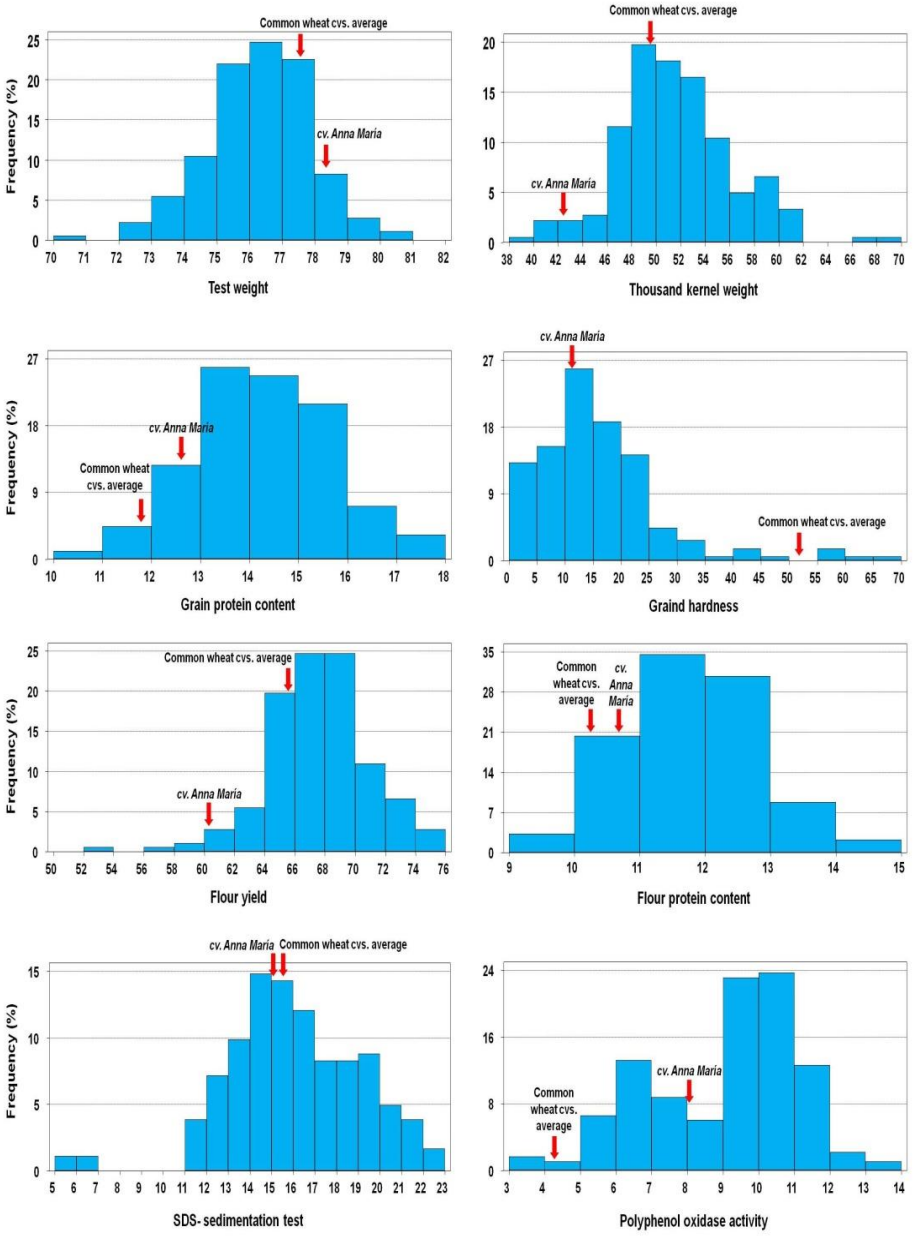

Figure 1. Frequency distribution of the spelt genotypes and average values of the common wheat cultivars for different grain and flour quality traits.

For the PPO activity, the range was very wide (3-14 $Ug^{-1}min^{-1}$), and two groups of materials can be distinguished among the spelt genotypes: one with a mean value of 6.5 $Ug^{-1}min^{-1}$, and another with the mean values around 10.5 $Ug^{-1}min$.

Alveogram and baking traits in the spelt collection

In the previous comparison with common wheat (Table 1), the data showed that spelt had doughs with low tenacity, high extensibility, and with low to medium gluten strength, as indicated by their W values. The analysis of the 80 genotypes that could be evaluated with the alveograph, showed a wide variation for the traits measured with this equipment (Table 2 and Table S3), with different genotypes exhibiting values higher or lower than the average. In this respect, some spelt genotypes could be classified as medium to high gluten strength with W up to 388×10^{-4} J. Spelt genotype BGE 020900 ($W = 320 \times 10^{-4}$ J in average across the two years) could be considered a good donor of this trait for breeding programs. For gluten extensibility, several spelt accessions (such as BGE 001990, PI 348727, or PI 348747) showed very low P/L values (0.3), and could be considered interesting sources of this trait. Accession PI 348465 showed a very interesting combination of both gluten strength and extensibility ($W = 283 \times 10^{-4}$ J and P/L = 0.4) and could be considered the best material found in terms of gluten quality. The modern spelt (cv. Anna Maria) presented values around the average values of the spelt set (Table 2). In this case, the ANOVA analysis showed also the high influence of the genotype in the variation detected (Table S4).

Table 2. Comparison of the alveograph parameters obtained in the traditional spelt accessions and the modern spelt cultivar Anna Maria.

	Traditional spelt		cv. Anna Maria
Trait	Mean ± s.d.	Range	Mean ± s.d.
P (mm)	59.48 ± 16.58	29.00-138.00	55.00 ± 4.24
L (mm)	123.87 ± 23.31	55.00-186.00	120.50 ± 17.67
P/L (ratio)	0.51 ± 024	0.20-1.70	0.45 ± 0.02
P/G (ratio)	2.47 ± 0.90	1.00-6.70	2.30 ± 0.00
W (x 10^{-4} J)	186.49 ± 57.18	73.00-388.00	192.00 ± 62.22
Ie (%)	46.04 ± 6.42	28.40-63.20	51.20 ± 10.32

P, dough tenacity; L, dough extensibility; G: swelling index; W, dough strength; and Ie, elasticity index.

As already mentioned, the mean values between spelt and common wheat for loaf volume did not show significant differences (Table 1). However, when the 50 genotypes of the spelt set were independently analysed, these materials exhibited a high variability for this trait, with minimum and maximum values of 600 and 975 cc, respectively (Figure 2). Apart from that, almost 82% of these genotypes had loaf volume between 750-875 cc. Genotypes PI 469058 and PI 469051 (885 mL and 848 mL of loaf volume, respectively in average across the two years) were the best performers for this trait and could be used by breeding programs aiming the improvement of bread-making quality.

Finally, a correlation analysis was carried out with the analyzed traits (except loaf volume, due to the lack of this data in many genotypes) within the spelt wheat set (Table S5). A negative correlation was found between test weight and protein content and grain size (TKW) and alveograph W. Positive correlations were identified between protein

content and SDS-sed and gluten extensibility (L), and among the different alveograph parameters.

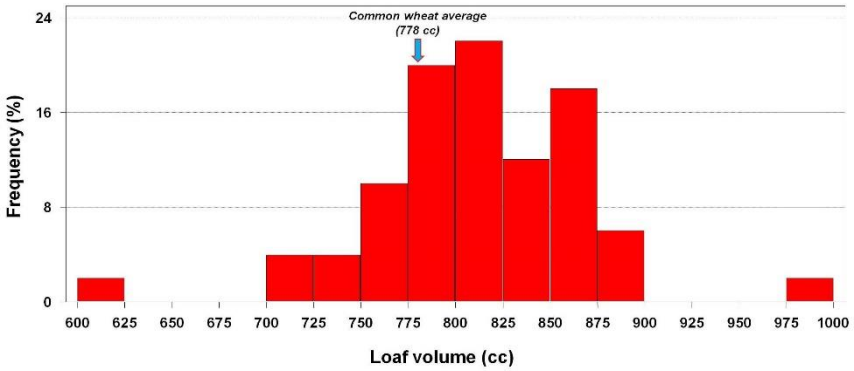

Figure 2. Frequency distribution of the spelt genotypes and average values of the common wheat cultivars for loaf volume.

Discussion

The changes in agri-food have generated a growing interest in foods and old crops that have been practically lost during the last century (Padulosi *et al.*, 2002). In some cases, this renaissance has been associated with the supposed miraculous properties of these old crops. In general, such statements are not supported by any scientific basis and both the nutritional or nutraceutical properties of the ancient wheat have been showed to be very similar to those of modern wheat (Miedaner and Longin, 2016; Cooper, 2015; Arzani and Ashraf, 2017; Bordoni *et al.*, 2017; Dinu *et al.*, 2018; Shewry, 2018; Brouns *et al.*,2022) However, other real benefits are little appreciated, such as the expansion of diversity in food.

Within the wheat world, both the old varieties, that were replaced by more productive ones, and some of the species that were cultivated in the past have been recovered during the last decades (Geisslitz *et al.*, 2020; Miedaner *et al.*, 2016). Some of the latter are called «ancient wheats» and consists mainly of three species: einkorn (*T. monococcum* L. ssp. *monococcum*, 2n = 2× = 14, A^mA^m), emmer and spelt. Some of the agronomic characteristics of these ancient wheats were those that led to their disappearance and abandonment when most of the agricultural processes were mechanized. In addition, due to being hulled grain cereals, the need of a special dehulling treatment prior to grinding increase costs and affect their profitability. For this reason, their revival is linked to the boom of the traditional and gourmet bakeries where the higher prices of these products can offset production costs. Nowadays larger retailers offer also flour and products done with these types of wheats.

At the same time, this renewed interest has resulted in the development of numerous studies on these species, comparing their characteristics with the ones of modern wheat (Shewry, 2018; Brouns *et al.*, 2022). However, many of these studies have been carried out with a limited number of genotypes (Shewry, 2015), which could bias the results and undervalued the true role of these old materials. For this reason, the evaluation of large collections of these ancient wheats, with the limitation of the storage materials in Germplasm Banks, could shed light on these questions, identifying new genotypes with potential utility for breeding programs. In the current study, one collection of 88 Spanish

traditional spelt accessions, together with nine common wheat cultivars and one modern spelt cultivar (cv. Anna Maria), were analyzed and compared among them for several traits related with the milling, processing, and end-use quality.

When the analyzed materials are ancient or old wheats, the technological quality must be evaluated with caution. Changes in baking techniques throughout the last century generated materials adapted to these techniques, different from traditional baking, and, consequently, the evaluation of ancient wheat according to modern parameters could not be the best strategy. In this regard, our previous studies on the grain composition of the spelt accessions evaluated in the current study showed the presence of rare HMWGs variants in spelt wheat (1, 13+16, 2+12) (Caballero, *et al.,* 2001; Caballero *et al.,* 2004). However, it is possible that the high frequency of these variants is an empirical consequence of the way these wheats were and are used in traditional agri-food. These characteristics are mainly demanded by bakers, since all the studies carried out suggest the clear influence of glutenins on the viscoelastic properties of wheat dough (Wrigley, *et al.,* 2006). However, millers are interested more in other traits more related to the physical and chemical characteristics of the grain such as TW, TKW, protein content or grain hardness, mainly due to their influence on the flour yield.

Previous studies have showed that the grain size in spelt is larger for the modern material (with common wheat introgression) than for the traditional spelt (Winzeler *et al.,* 1994; Ratajczak *et al.,* 2020; Kulathunga *et al.,* 2021). In this study, the traditional spelt genotypes

presented a TW and TKW very similar to the common wheat cultivars using as control, and, compared with the modern spelt (cv. Anna Maria), this latter has a better TW but its TKW was sensitively lower. This reinforces the idea that the variation in the traditional spelt is high and could be interesting for breeding programs aiming to develop new cultivars with very high grain size (Packa *et al.,* 2019; Guzmán and Alvarez, 2021). In parallel, the spelt genotypes with high grain size showed high protein content (indeed, a significant correlation between TKW and protein content was found) and a soft texture, which positively affected flour yield.

The PPO activity is appears comparatively higher in spelt than in common wheat. This trait is regulated by several enzymes, synthesized by the *Ppo-1* and *Ppo-2* loci at the homoeologous group 2 chromosomes (Demeke and Morris, 2002; Jukanti *et al.,* 2004; He *et al.,* 2007; Beecher and Skinner, 2011; Taranto *et al.,* 2015) and has been associated with the discoloration and darkening of the wheat products (Anderson and Morris, 2003; Baik *et al.,* 1995; Demeke *et al.,* 2001; Fuerst *et al.,* 2006), which generates certain rejection among the consumers (Anderson and Morris, 2001; Jukanti *et al.,* 2004; Baik *et al.,* 1995; Demeke *et al.,* 2001; Fuerst *et al.,* 2006; Feillet *et al.,* 2000). Paradoxically, it may happen that today´s consumers associate this dark color to the true presence of flour spelt in a food product, while the cream color suggests that the product is made with flour of modern common wheat and not from spelt (more "natural" vs. more "industrial"). Therefore, high PPO activity may not be an undesirable trait for spelt cultivars, although it should deserve

attention if spelt will be used in the breeding of modern common wheat as source of other traits of interest. In this regard, some traditional spelt genotypes showed low PPO activity values (≤ 5 $Ug^{-1}min^{-1}$) (Table S2), although this is not the general trend.

Previous studies conducted on spelt, revealed that spelt mostly exhibit low to medium gluten strength (Abdel-Aal *et al.*, 1997; Bojnanská and Francáková, 2002; Wilson *et al.*, 2008; Ratajczak *et al.*, 2020; Podolska *et al.*, 2020; Kulathunga *et al.*, 2021; Hadnadev *et al.*, 2022) however, our study reveals that it is also possible to identify genotypes with stronger gluten. In any case, the viscoelastic properties of spelt could be different from those of common wheat. The spelt genotypes showed in general more extensible doughs, which was favored by higher protein contents, and in few cases, the W values were reasonably high (up to 300 x 10^{-4} J). This was also due to the strong correlation found between alveograph W and P/L, which is also normal in common wheat sets (Guzmán *et al.*, 2022). However, it was not possible to identify an unambiguous relation between the W values and loaf volume. As with other measured traits, the variation of these two parameters was high among the spelt genotypes. When the loaf volume was related with the flour protein content, some genotypes with low protein content showed high loaf volume, which suggests the high quality of these gluten proteins. The current trend in the cereal's world has extended the search for other desirable traits within the grain components, mainly related with nutritional and nutraceutical properties, which would complement the technological properties of the doughs

(Shewry, 2005; Gomez-Becerra, *et al.,* 2010; Bouis *et al.,* 2011; Jones *et al.,* 2020). Nowadays, the presence of micronutrients as Fe or Zn in the flour, or dietary fiber in form of soluble- arabinoxylans is highly recommendable and it increased the interest for the ancient wheats, where some studies suggest that these old materials could be a good source for these traits (Ranhotra *et al.,*1996; Guzmán *et al.,* 2014; Ruibal-Mendieta *et al.,* 2005).

In this respect, a previous study on these nutritional aspects has revealed that these current spelt genotypes show a notable variation for these traits (Huertas-García *et al.,* 2023). These data, together with the data obtained in the current study, stressed the importance to increase the evaluation of wide collections of these ancient wheats, in order to detect the true variability presents in these old materials for different traits, including those associated with processing and nutritional quality. Such analyses will allow the identification of unique germplasm that could be used both for the selection and purification intra-accession destined to the development of traditional and homogeneous spelt varieties, both to be crossed with modern wheat to transfer the trait of interest, to improve modern wheat genetic diversity, or to develop better adapted spelt cultivars.

Conclusions

Ancient wheats can be good sources of interesting agronomic, mainly rust resistant genes, and quality traits for wheat breeding. The evaluation of the large collections of these old materials would allow

evaluating the true variability present in these species. In the current study, large variation was found in a set of Spanish spelt landraces, which in general showed soft grain, medium-high protein content, low gluten strength, high gluten extensibility, and medium bread-making quality; spelt genotypes showing outstanding values for some of these traits that could be useful for breeding purposes were identified. Additionally, this and similar studies could open the opportunity to develop new cultivars of spelt with good characteristics for the food industry.

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DISCUSIÓN GENERAL Y CONCLUSIONES

En trigo, la calidad del grano es uno de los factores más importantes del cultivo, ya que determina su valor de venta en el mercado y su posterior uso. Esta calidad viene determinada por la composición del grano y por las características derivadas de la misma, como son la dureza, y la fuerza del gluten. La calidad nutricional está relacionada con la concentración de algunos componentes del grano entre los que destacan el hierro, zinc, ácido fítico y la fibra por su importancia en el metabolismo y en la salud humana.

Dentro del mundo del trigo, tanto las variedades antiguas, que fueron sustituidas por otras más productivas, como algunas de las especies que se cultivaban antiguamente se han recuperado durante las últimas décadas (Geisslitz *et al*., 2020; Miedaner *et al*., 2016). Algunos de estos últimos se denominan «trigos antiguos». En el pasado, estos trigos milenarios fueron descuidados principalmente por su menor rendimiento, su mala adaptación a la mecanización agrícola y porque requerían un tratamiento especial de descascarillado en el molino para separar la paja del grano. Actualmente, su mayor interés está más relacionado con su uso en alimentación, debido a un creciente interés por alimentos y cultivos tradicionales que prácticamente se han perdido durante el último siglo.

Los trigos antiguos tienen interés como cultivo per se y como donantes de características, ya que poseen variabilidad para diferentes genes que pueden no estar presentes en los cultivares modernos. Dicha

variabilidad podría ser útil para que los programas de mejora modernos centrados en el desarrollo de materiales con características de calidad diferenciales y más nutritivos y el desempeño agronómico de los trigos modernos (Padulosi *et al.,* 2002). Es por ello que en la actualidad se está potenciando la búsqueda de nueva variabilidad genética en los recursos genéticos disponibles almacenados en los Bancos de Germoplasma. Al mismo tiempo, se están desarrollando numerosos estudios sobre los trigos antiguos, comparando sus características con las del trigo moderno (Shewry, 2018; Brouns *et al.,* 2022). Sin embargo, muchos de estos estudios se han realizado con un número limitado de genotipos (Shewry, 2015), lo que podría sesgar los resultados e infravalorar el verdadero papel de estos materiales antiguos. En consecuencia, la evaluación de colecciones más grandes es fundamental, y esto ha motivado el desarrollo de estudios más amplios (Curzon *et al.,* 2021; Tóth *et al.,* 2022), ya que la evaluación de grandes colecciones de estos trigos antiguos podría arrojar luz sobre estas cuestiones, identificando nuevos genotipos potenciales para utilizarlos en programas de mejora.

En esta Tesis Doctoral se ha evaluado la variabilidad para diferentes características de calidad y genes relacionados con la misma en diferentes recursos genéticos de trigo, incluyendo especies silvestres, y variedades locales de trigo harinero y de espelta. La variación genética fue caracterizada molecularmente en algunos casos para establecer las diferencias entre las distintas variantes alélicas, y así intentar inferir su repercusión en las características de calidad relacionadas.

En primer lugar, se analizó una colección de 170 accesiones de

escaña silvestre, un trigo diploide que se ha clasificado como ancestro del primer trigo cultivado, para ver su variabilidad en los loci *Glu-A^{m}1*, *Wx-A^{m}1* y *Ha*. Se detectó una amplia variabilidad para los genes estudiados lo que sugiere el potencial interés de esta especie para utilizarse en programas de cruzas amplias como fuente de nuevos alelos.

A continuación, se procedió con el estudio de una colección de 271 variedades locales de trigo harinero iraní. Estos materiales fueron seleccionados en base a estudios previos, en los que los granos se habían clasificado como duros o semiduros en función de su dureza. Eran, por tanto, materiales ideales para estudiar en los mismos la variabilidad de los genes *Pina-D1* y *Pinb-D1*, con el fin de detectar nuevos alelos de estos genes asociados a diferentes durezas del grano. Se detectaron varios alelos ya descritos en otros trabajos y un nuevo alelo caracterizado por el cambio de una cisteína por una tirosina en la secuencia de la proteína deducida. Este alelo podría estar asociado a diferentes niveles de dureza y podría ser utilizado por los programas de mejora interesados en la afinación de dicho carácter.

Para el estudio de componentes relacionados con la calidad nutricional se realizó un ensayo de campo durante dos años con una colección de ochenta y ocho accesiones de trigo espelta de origen español, junto con nueve cultivares de trigo harinero y uno de espelta modernos. Se detectó una amplia variabilidad para diferentes componentes del grano con importancia nutricional (fibra, hierro, zinc, y ácido fítico) en ambas especies y se identificaron algunos genotipos con valores extraordinarios que podrían ser utilizados en programas de

mejora específicos y para estudios genéticos más profundos, enfocados en identificar el control genético de estas características. Además, los materiales de este ensayo se utilizaron también para evaluar en profundidad la calidad de procesamiento y panificación, encontrándose interesantes características en algunas accesiones de espelta que podrían utilizarse en la mejora de dicha especie.

A partir del trabajo realizado se han obtenido las siguientes conclusiones:

1. La escaña silvestre es una fuente interesante de nuevas variantes de genes relacionados con la calidad del trigo.

2. Una colección de variedades locales de trigos harineros iranís mostró una notable variabilidad para los genes de las puroindolinas. Se detectó un nuevo alelo para el gen *Pinb-D1* (*Pinb-D1ak*), el cual posee un cambio en la estructura de la proteína, lo que provoca que los granos de estos materiales sean duros. Futuros estudios determinarán si el nivel de dureza asociado a este alelo es diferencial del de otros alelos de puroindolinas.

3. Entre trigo espelta y trigo harinero existe una variación intra e interespecífica significativa en los compuestos nutricionales, por lo que no se puede afirmar que una especie sea más saludable que la otra. Dentro de ambos grupos

existen genotipos con valores sobresalientes para determinados compuestos nutricionales, que podrían utilizarse como donantes en los programas de mejora.

4. Existe una gran variación para características de calidad de procesamiento y panificación en la colección de espelta español. En general dichos materiales poseen grano blando, contenido proteico medio-alto, baja fuerza y alta extensibilidad del gluten, y calidad panadera media. Se identificaron genotipos con valores sobresalientes en algunos de estos rasgos que podrían ser útiles para la mejora genética y para desarrollar nuevos cultivares de espelta con buenas características para la industria alimentaria.

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MATERIAL SUPLEMENTARIO

El material suplementario de esta tesis doctoral se encuentra disponible a través de este código QR. Para acceder a dicho material escanee este código QR y podrá descargar el PDF con todo el contenido.

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