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VARIABILIDAD PARA PROTEINAS DE RESERVA DE TRIGO ESPELTA DE ORIGEN ESPAÑOL

TESIS DOCTORAL

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INFORMAN:

Que el trabajo titulado: **"Variabilidad para proteinas de reserva de trigo espelta de origen español"**, realizado por Da. Leonor Caballero García, bajo su dirección, se considera ya finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

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Introducción general

Importancia de la Agricultura

Una de las situaciones más trascendentales acontecidas en la historia de la Humanidad es sin duda la aparición de la Agricultura, la cual marca un punto de inflexión que ha cambiado radicalmente las relaciones del Hombre con la Naturaleza. Previo a este acontecimiento, se desarrolla la mayor parte de la evolución de la especie (≈ 2 millones de años), seguido de un periodo que ha sido denominado como "fase salvaje" o "era de la barbarie" en la cual se desarrollan las actividades de cazador-recolector, similares a las que subsisten en algunas tribus del Amazonas. A pesar del claro sentido peyorativo de la definición que se atribuye a Lewis Henry Morgan, uno de los fundadores de la Antropología, durante este periodo, se desarrolla un progresivo conocimiento de las plantas y los animales que serán fundamentales en el desarrollo de la Agricultura y la Ganadería (Harlan, 1992).

Hacia finales de este periodo, en un proceso denominado como "*Revolución Neolítica*", comienza a desarrollarse la Agricultura. La cronología ha sido diferente en cada uno de los principales centros de origen establecidos por Nicolaiv I. Vavilov en 1930 (Vavilov, 1992), dado que la cronología basada en Edades se fundamenta en la adquisición de determinados usos de los materiales (piedra o metales) y no en fechas concretas. En el Próximo Oriente, este proceso comienza a desarrollarse en torno a los 10.500 años a.C. en la zona conocida como *Creciente Fértil* que incluye parte de las actuales Siria, Turquía, Irak e Irán, en lo que sería la zona de Mesopotámica (valles de los ríos Tigris y Eúfrates). Este proceso fue muy posterior en el Extremo Oriente (Península de Indochina) donde aparece entre el 6.500-5.500 a. C., o en América Central y Andina entre el 5.000-4.000 a. C.

No se conoce con certeza qué llevó al Hombre a abandonar los métodos tradicionales de subsistencia y dedicarse a la Agricultura, aunque Mannion (1999) propone dos posibles hipótesis: una materialista y otra medioambientalista. La primera se basa en el aumento demográfico, que trajo consigo cambios sociales como el sedentarismo, el deseo de producir más alimentos como medida de seguridad y de poder, para mantener un estilo de vida en esta nueva sociedad. La medioambiental se basa en los cambios climáticos ocurridos tras la última glaciación, la cual provocó un impacto ecológico, redistribuyendo los recursos vegetales y animales. Aunque el resultado muy posiblemente, proceda de la interacción de ambas, la consecuencia es la misma, una vez dado el paso hacia el sedentarismo no hay vuelta atrás.

El nacimiento de la Agricultura trajo consigo un cambio social y cultural de la Humanidad, supuso cambios drásticos e irreversibles como el sedentarismo y la especialización en el trabajo, que unidos a la explosión demográfica, llevó paulatinamente al establecimiento de estructuras de población cada vez más complejas como son la Ciudad, el Estado y el Imperio, proporcionando todos los elementos sustanciales de la Sociedad que conocemos.

Entre las primeras plantas domesticadas por el Hombre hay que destacar a los cereales y leguminosas, lo cual posiblemente esté asociado con su elevado valor calórico y facilidad de conservación que les permitía una mayor disponibilidad de ellos en el tiempo, así como a la complementación nutricional que presentan. De entre los cultivos más antiguos de los que se tienen constancia (Zohary, 1992) se encuentran: el einkorn (2n=2x=14; **AA**; *T. monococcum* ssp. *monococcum* A. et D. Löve), el emmer cultivado (2n=4x=28; **AABB**; *Triticum turgidum* ssp. *dicoccum* Schrank), y la cebada (2n=2x=14; **II**; *Hordeum vulgare* L.).

El trigo, su origen e importancia en la Cuenca Mediterránea

Los cereales han sido el principal cultivo en la mayoría de las civilizaciones. Las civilizaciones mediterráneas se basaron en el trigo y la cebada,

las de Asia en el arroz, las de África en el sorgo y las de América en el maíz. Sus granos constituyeron la mayor fuente de calorías en la alimentación humana. Los primeros signos del cultivo del trigo y la cebada aparecieron en el Próximo Oriente hacia finales de 8º milenio a.C. La domesticación de estas especies hizo posible la expansión de la agricultura neolítica desde este centro al oeste de Asia, Europa y norte de África, desde la costa Atlántica a la India subcontinental y desde Escandinavia al Valle del Nilo.

El trigo es un complejo poliploide, cuya importancia y uso a lo largo de la Historia ha ido cambiando considerablemente. Las teorías más aceptadas actualmente en cuanto a su origen y evolución, sugieren que todas las especies de Triticíneas proceden de una especie ancestral de siete pares de cromosomas, que ha dado lugar a tres grupos según su nivel ploidico: diploide, tetraploide y hexaploide.

T. monococcum ssp. *aegilopoides* Link em. Thell (syn. *T. boeoticum*) es el trigo silvestre que se conoce desde más antiguo; originario del Creciente Fértil, esta especie diploide fue domesticada por el hombre dando lugar a *T. monococcum* ssp. *monococcum* L. o einkorn, también conocido como escaña. Otra especie diploide es *T. urartu* Thum. En lo referente al trigo tetraploide, la especie silvestre es *T. turgidum* ssp. *dicoccoides* L. (**AABB**). La domesticación de este trigo tetraploide silvestre dio lugar a *T. turgidum* ssp. *dicoccum,* del que evolucionaron posteriormente otras subespecies de trigos duros, *T. turgidum* ssp. *durum* (Desf.) MacKey, *T. turgidum* ssp. *turgidum* L. y *T. turgidum* ssp. *carthlicum* A. et D. Löve. Otra especie tetraploide es *T. timophevii* Zhuk (**AAGG**) que incluye tanto a formas silvestres (*T. timophevii* ssp. *araraticum*) como cultivadas (*T. timophevii* ssp. *timophevii*). Esta especie es endémica de la región de Georgia, URRS y parece representar un episodio local en la evolución del trigo. No obstante, hay estudios que avalan que el genoma **A,** tanto de los trigos tetraploides como de los hexaploides, procede del *T. urartu* (Nishikawa, 1984; Dvorak, 1988).

Se desconoce el origen del genoma **B**, aunque hay pruebas que sugieren la posibilidad de que *Aegilops speltoides* Tauch u otra especie de la sección *Sitopsis*, sea el donador de dicho genoma tanto a los trigos duros como harineros (Dvorak &

Zhang, 1990, 1992; Daud & Gustafson, 1996). Aunque esto no está claro en el caso de *T. timophevii* Zhuk (**AAGG**) (Feldman, 1966).

En trigos hexaploides, espelta (2n=6x=42; **AABBDD**; *T. aestivum* ssp. *spelta* L. em Thell.) sería el trigo hexaploide primitivo del que habría evolucionado el trigo harinero moderno (*T. aestivum* ssp. *aestuvum* L. em. Thell). Este trigo se habría producido por el cruzamiento espontáneo entre *T. turgidum* ssp. *dicoccum* y *Ae. tauschii* ssp. *strangulata* Coss. donador del genoma **D** (McFadden & Sears 1946; Keber & Rowland 1974), muy posiblemente en la zona del mar Caspio donde se distribuye esta especie diploide. Cuando se sintetiza artificialmente el *T. aestivum* por cruzamiento entre *T. turgidum* y *Ae. tauschii* el producto resultante es un trigo vestido como espelta, independientemente de que el parental tetraploide sea vestido o desnudo (Keber & Rowland 1974).

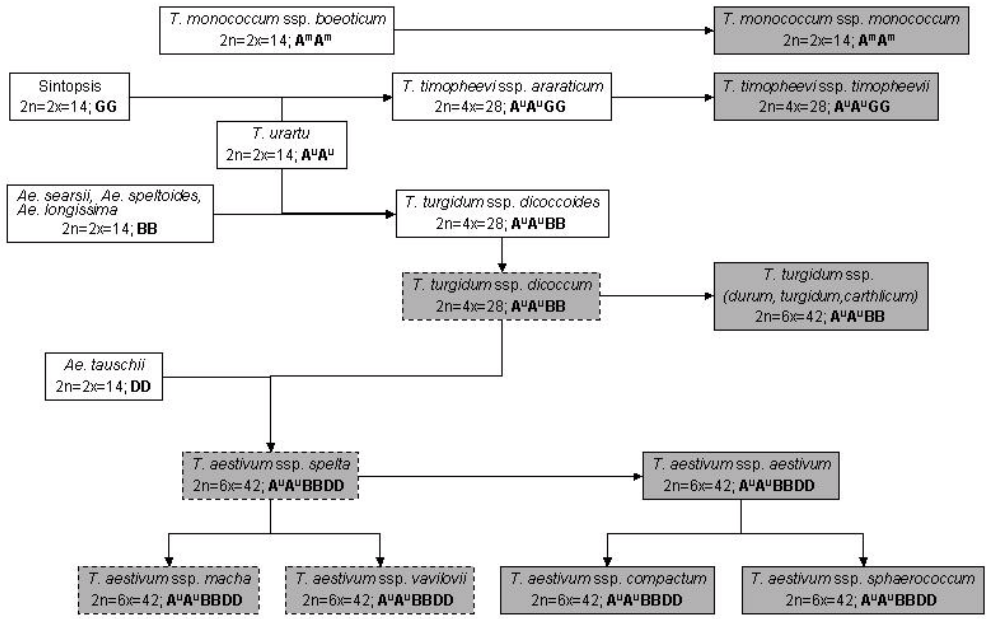

Figura 1.- Origen y evolución del trigo. Las especies sombreadas son o han sido cultivados, con línea discontinua aparecen aquellos que son de grano vestido.

La panificación

Uno de los usos más importantes del trigo es su capacidad de producir pan. Las primeras referencias escritas sobre el conocimiento del pan, se remontan a 2600 años a.C. Si bien, los hallazgos arqueológicos más antiguos indican que el pan ya se conocía en Babilonia 4000 años a.C. Los egipcios fueron los primeros en utilizar la levadura para provocar la fermentación y esponjamiento de la masa de harina de trigo. Utilizaban el pan como un emblema del estatus social que ocupaban, las clases humildes comía un pan tosco y da baja calidad, mientras los sacerdotes y los altos cargos del Estado, incluido el Faraón, tomaban panecillos realizados con la mejor harina (Harlan, 1981; Pomeraz, 1987; Orht & Shellenberger, 1988).

Siguiendo el proceso histórico, los conocimientos sobre panificación pasaron de los egipcios a los griegos y de estos a los romanos. Los griegos preferían tomar la harina en forma de gachas. En cambio, para los romanos, el pan tuvo su importancia desde el punto de vista cultural y religioso, potenciaron su distribución en la cuenca mediterránea y establecieron su fabricación a nivel industrial (dado el progresivo aumento del tamaño de las ciudades), al igual que los egipcios utilizaron el pan como un símbolo de la posición social que ocupaban, así generaron una gran variedad de panes:

- *panis candidus*, era el pan blanco, hecho con la mejor harina. De aquí deriva la definición de pan candeal, sinónimo de pan de alta calidad.
- *panis secundarius*, era el pan normal.
- *panis acerous, plebeius, rusticus, castrensis o sordidus,* era el pan barato,de escasa calidad.

Los romanos desarrollaron las panaderías a escala industrial, fundando la primera organización de panaderos o gremio de panaderos, el *collegium pistorum*, nombre derivado de *pistores* que significa golpeadores, ya que el modo en que se limpiaba los trigos vestidos era golpeándolos en un mortero. De todas formas gran parte del pan que se consumía seguía haciéndose en los propios hogares.

En la Revolución Industrial se consiguió la mecanización de muchos de los procesos y se produjo un gran despegue de la industria panificadora. En este

momento se desarrollan nuevos y diferentes productos, usando diferentes materias primas e incluso otros cereales. El aumento de la población provocó la búsqueda de variedades más rendidoras y la mecanización de los procesos de panificación la de variedades cuya harina formase una masa que ofreciera tolerancia al amasado y sobreamasado mecánico.

En nuestros días el trigo tiene diversos usos en la industria de los alimentos siendo el ingrediente principal en la elaboración de pan, galletas, productos de repostería y pastas. En cuanto a la calidad del trigo, dependería de las preferencias del consumidor, del producto que se quiera elaborar, del proceso que se va a utilizar y el éxito de un producto en el mercado. En el caso particular de la calidad harino-panadera del trigo, ésta depende de las propiedades reológicas del gluten que se obtiene a partir de la harina, que, a su vez, depende de las proteínas de reserva del endospermo. En el trigo, dichas proteínas poseen la propiedad distintiva y única de formar gluten cuando se mezcla harina con agua y se le aplica un trabajo mecánico. El gluten se ha definido como "la estructura viscoelástica que queda tras el lavado del almidón de una masa formada con harina de trigo" (Miflin et al., 1983). Las diferencias entre variedades para la calidad del gluten son causadas por las diferentes combinaciones alélicas de las proteínas de reserva. Este componente genético puede ser manipulado y mejorado. No obstante, la calidad está directamente influenciada por los factores climáticos que el cultivo pueda sufrir en el campo, condiciones del suelo, estrés, los manejos de cultivo, cosecha, almacenamiento, así como por otros factores no controlables por el productor como: condiciones de transporte, procesado del grano y su reducción de harina.

Proteínas de reserva: su relación con la calidad y su aporte en los estudios de diversidad

Los componentes del endospermo del grano de trigo en orden de importancia son: hidratos de carbono, proteínas, lípidos, y otros componentes menores como vitaminas y trazas minerales (Aykroyd & Doughty, 1970), siendo las proteínas de reserva las de mayor importancia en la panificación (Pomeraz, 1988).

Se han realizado varios intentos de clasificación de las proteínas, entre los que cabe destacar el desarrollado por Shewry et al., 1986 basada en sus características biológicas y en sus relaciones químicas y genéticas. Así, las dividió en: grupo de alto peso molecular (que corresponde a las subunidades de alto peso molecular de glutenina), grupo de prolaminas pobres en azufre (ω-gliadinas) y grupo de prolaminas ricas en azufre (γ-, β-, α-gliadinas y las subunidades de bajo peso molecular de glutenina). Sin embargo, en la actualidad, la única aceptada por la comunidad científica internacional es la desarrollada por Osborne en 1924. Esta se basa en la solubilidad de las proteínas en distintos solventes, pudiendo distinguirse de este modo cuatro fracciones:

- albúminas, constituidas por las proteínas solubles en agua;
- globulinas, solubles en soluciones alcalinas diluidas (0.5M de NaCl);
- prolaminas, solubles en soluciones alcohólicas;
- glutelinas, solubles en ácidos o alcalis diluidos.

Las dos primeras fracciones incluyen a las proteínas que tienen funciones fisiológicas, es decir, a las enzimas que controlan el proceso germinativo. En la panificación un exceso de estas fracciones resulta negativo dado que destruyen toda la estructura interna de la masa provocando una pérdida de CO_2 y una falta de homogeneidad en la textura interna del pan.

La fracción de prolaminas constituye las proteínas de reserva que en trigo se denominan gliadinas y gluteninas, y que en el proceso germinativo aportan los aminoácidos necesarios para el desarrollo del embrión.

Las gliadinas tienen un tamaño, entre 30 y 75 kDa, su estructura es monomérica y se asocian por puentes de hidrógeno e interracciones hidrofóbicas. Cuando se fraccionan en sistema de electroforesis a pH ácido en tampón lactato de aluminio, presentan cuatro grupos distintos en función de su movilidad con respecto al cátodo: ω-, γ-, β- y α-gliadinas. Las β- y α-gliadinas tienen un peso molecular de 30 a 36 kDa y presentan 6 residuos de cisteína, que permiten la formación de puentes disulfuro intra- e intermoleculares (Wrigley & Beitz, 1988; Sherry & Tatham, 1997). Las γ-gliadinas presentan 8 residuos de cisteína, todos ellos situados

en el dominio C-terminal, y su peso molecular varía entre 38 y 42 kDa (Sherry & Tatham, 1997). Las ω-gliadinas tiene un peso molecular mayor, entre 44 y 75kDa, y son diferentes con respecto a los otros grupos de gliadinas, tanto en su composición de aminoácidos como en sus secuencias de aminoácidos en el dominio N-terminal, que no contiene residuos de cisteína. (Booth & Ewart, 1969; Charbonnier, 1974; Charbonnier et al., 1980; Masci et al., 1991).

La mayoría de las gliadinas están controladas por los loci complejos *Gli-1* y *Gli-2* situados en el brazo corto de los cromosomas de los grupos homeólogos 1 y 6, respectivamente (Payne et al., 1982a). Cada locus complejo que codifica gliadinas consiste en varios genes estrechamente ligados y el resultado de los mismos aparece como un conjunto de proteínas que suelen ser estudiadas como un bloque, el cual se considera como una variante alélica (Sozinov & Poperelya, 1980). Las gliadinas están relacionadas con las propiedades viscoelásticas del gluten. No obstante, su principal uso ha sido la identificación varietal, ya que presentan un elevado nivel de polimorfismo, por lo que el patrón electroforético de cada variedad suele ser único (Lee & Ronalds, 1967; Autran & Bordet, 1975; Zillman & Bushuk, 1979; Dal Belin Peruffo et al., 1981).

El otro grupo de proteínas de reserva, gluteninas, son de gran tamaño, de 100 kDa hasta varios millones de daltons; de carácter polimérico, están formadas por subunidades unidas por puentes disulfuro (Huebner & Wall, 1976). Basándose en el peso molecular, las subunidades se dividen en: subunidades de alto peso molecular y subunidades de bajo peso molecular (Payne et al., 1984). Las gluteninas pueden clasificarse también en cuatro grupos, denominados A, B, C y D (Jackson et al., 1983). El grupo A corresponde a las subunidades de alto peso molecular, mientras que los grupos B, C y D incluyen a las subunidades de bajo peso molecular, quedando el grupo D en una posición intermedia entre los grupos A y B.

Las subunidades de alto peso molecular son sintetizadas en un locus complejo denominado *Glu-1* presente en el brazo largo de cada uno de los cromosomas del grupo homeólogo 1, denominándose *Glu-A1*, *Glu-B1* y *Glu-D1*, respectivamente (Biezt et al., 1975; Lawrence & Shepherd, 1981, Payne et al., 1982b). A su vez cada locus está formado por dos genes estrechamente ligados,

cada uno de los cuales sintetiza una subunidad, que presentan distinta movilidad electroforética denominada tipo *x* (la más lenta) y tipo *y* (la más rápida). Los genes que codifican para las subunidades de bajo peso molecular de glutenina, loci *Glu-3*, están estrechamente ligados con los loci *Gli-1* que codifican para ω- y γ-gliadinas y se encuentran en el brazo corto de los cromosomas del grupo homeólogo 1 (Jackson et al., 1983). Así los loci *Glu-A3*, *Glu-B3* y *Glu-D3* se encuentran en los cromosomas 1A, 1B y 1D, respectivamente (Singh & Shepherd, 1988). En el proceso de panificación, las gluteninas, en su conjunto, confieren la elasticidad a la masa.

Los estudios efectuados por Payne et al. (1982) y confirmados posteriormente indican que el locus *Glu-1* juega un importante papel en la calidad harino-panadera.

La base molecular de las diferencias en calidad que confieren las distintas subunidades de alto peso molecular está aún en investigación. La secuenciación de ADN de estos genes ha revelado algunas características de este tipo de proteínas. Un rasgo importante de las subunidades de alto peso molecular de glutenina es el número y distribución de los residuos de cisteína, ya que juegan un papel muy importante en la formación de polímeros de glutenina (Lafiandra et al., 1995; Lindsay et al., 2000). En general, en la región N-terminal de las subunidades de tipo *x* y de tipo *y*, existen 3 y 5 residuos de cisteína, respectivamente, mientras que en la región C-terminal está presente un solo residuo en ambos tipos de subunidades. Así, se ha sugerido que la mayor calidad harino-panadera asociada a las subunidades 5+10 frente a las subunidades 2+12 se debe principalmente a la presencia de un residuo extra de cisteína en la subunidad Dx5 al inicio del dominio repetitivo central (Popineau et al., 1994; Greene et al., 1998; Lafiandra et al., 1993; Gupta & MacRitchie, 1994).

Además del locus *Glu*-1, es importante para la calidad la variación en las subunidades de bajo peso molecular de glutenina, gliadinas, así como las interacciones entre todos estos loci (Carrillo et al., 1990). Así, se han encontrado asociaciones entre determinadas subunidades de bajo peso molecular de glutenina y las propiedades de la masa en trigo harinero (Gupta & Shepherd, 1988; Gupta et al.,

1989; Khelifi & Branlard, 1992; Zhen et al., 1997). En trigo duro, se ha encontrado que un gluten fuerte está asociado más con el contenido en prolaminas ricas en azufre (γ-, β- y α-gliadinas y subunidades de bajo peso molecular de glutenina) que con las subunidades de alto peso molecular glutenina.

Las proteínas de reserva son muy útiles como marcadores, ya que presenta un elevado nivel de polimorfismo, que es de naturaleza genética, y que puede analizarse fácilmente mediante electroforesis. Además, todos los estudios sugieren su neutralidad frente a la selección neutral. Todo ello ha propiciado su uso como marcador genético en el análisis de la diversidad en muchas especies.

Los recursos genéticos

En los últimos años, las proteínas de reserva han recibido una gran atención como marcadores moleculares para el tratamiento de la variabilidad genética en las colecciones de germoplasma. Algunos autores consideran la electroforesis de proteínas de reserva de cereales en geles de poliacrilamida como la técnica más idónea para conseguir la caracterización de la variabilidad existente (Damania, 1990).

Como consecuencia de la domesticación, las plantas cultivadas han sufrido un proceso selectivo muy profundo que las ha hecho cambiar radicalmente respecto a las formas silvestres de las que se partía, a la vez que ha dado lugar a una gran variación genotípica por adaptación a distintos ambientes, manejos y usos. Así pues, aunque las formas silvestres poseen mayor variabilidad alélica, las formas cultivadas presentan mayor riqueza de genotipos. Este proceso de creación de variabilidad genética no se da en la actualidad.

La evolución de la Agricultura en los últimos 50 años, centrada en variedades de alto rendimiento, ha dado lugar a un estrechamiento de la base genética de los cultivos, ya que la necesidad de adaptarse a las condiciones de alto rendimiento que caracteriza a este tipo de agricultura conlleva que los materiales mejorados sean muy similares entre sí, a lo que hay que añadir la fuerte presión de

los nuevos cultivares obtenidos sobre a las variedades locales o tradicionales, hasta el punto de hacerlas prácticamente desaparecer o reducirlas a niveles puramente testimoniales. Este brusco proceso de evolución ha producido un efecto de deriva muy importante en los genes no directamente afectados por selección, como son los que regulan las proteínas de almacenamiento. Consecuentemente, una gran parte de la variabilidad genética existente para los genes de proteínas de reserva ha desaparecido de los campos de cultivo. Esta pérdida de variabilidad ha promovido la búsqueda de nuevas fuentes de variación que podrían ser usadas en los programas de mejora. Algunos podrían ser recuperados de los bancos de germoplasma existentes (Brown et al., 1989). Otra fuente de variación es el de las especies silvestres relacionadas con los cultivos o las especies cuyo cultivo ha desaparecido o se ha reducido drásticamente, pero de las que se conservan poblaciones en los bancos de germoplasma, como son los trigos vestidos.

Por lo tanto se hace necesario comenzar una exploración de los recursos genéticos contenidos en los bancos de germoplasma y su recuperación *per se* o a través de su incorporación a los cultivos actuales vía introgresión.

Los trigos vestidos a lo largo de la historia

En la Península Ibérica todavía es posible encontrar especies de cereales que fueron importantes durante la Prehistoria, pero en nuestros días están en grave peligro de extinción. Son los trigos vestidos, denominados así porque sus glumas permanecen adheridas al grano incluso tras la trilla. Otra característica de estos trigos es que poseen raquis semiquebradizo. Estas características vienen dadas por una serie de mutaciones en el gen Q, localizado en el cromosoma 5A (Luo et al., 2000).

Entre las especies de trigo vestido están: los trigos einkorn (*T. monococcum* ssp. *monococcum*), cuyo nombre común en Andalucía es la escaña; emmer cultivado (*T. turgidum* ssp. *dicoccum*), llamado povia, pavia, póvida y escanda menor en Asturias y ezkandia en Navarra, y espelta (*T. aestivum* ssp. *spelta*), llamada escanda mayor o fisga asturiana.

Como ya se ha mencionado, espelta es un aloploide producido por el cruzamiento entre emmer cultivado y *Ae. tauschii*, suceso que probablemente ocurrió en la región caspiana alrededor de los 6000 años a.C. Espelta junto con einkorn y emmer son los cereales más antiguos de Europa. En este continente principalmente en la región mediterránea y en el Próximo Oriente, estos cereales fueron populares durante cientos de años, constituyendo la mayor fuente de alimentación.

Los primeros antecedentes de los trigos einkorn y emmer en la Península Ibérica datan del Neolítico entre los años 3.00-3200 a.C. (Buxó & Capdevilla, 1993).

La evidencia arqueológica más temprana de espelta es en el 5º milenio a.C. en Transcaucasia, norte del mar Negro y norte de Irak. Según Nesbitt y Samuel (1996), las más abundantes y mejor documentadas evidencias arqueológicas de espelta en Europa fueron en sitios del Neolítico tardío (2500-1700 a.C.), donde se encontraron restos en el este de Alemania, Polonia y Jutlandia. Durante la Edad de Bronce se extendió ampliamente en el norte de Europa. En la Edad de Hierro (750-15 a.C.), espelta reemplazó a emmer como principal cultivo en el sur de Alemania y Suiza. Un patrón similar ocurrió en Inglaterra en el 500 a.C. y en el mismo periodo espelta estuvo presente en zonas del norte de España.

Aunque la especie es de origen iraní como previamente se ha indicado, el término *espelta* proviene del dialecto germánico y la primera referencia escrita donde aparece el nombre de espelta es el Edicto de Diocleciano del año 301 en el cual se establece el valor de las cosas. En España, los primeros datos históricos del cultivo de los trigos emmer y espelta se encontraron en el Cronicón Albendense (883 A.D.), en el que se habla de productos hechos con escanda asturiana (nombre genérico para ambos trigos).

Estos trigos vestidos jugaron un papel muy importante en los ritos y ceremonias religiosas, primero durante la Era Romana y luego durante el Cristianismo. En la Era Romana se usaban los términos *far, ador, semen adoreum, alica*, para definir a estas especies. Una de las formas de matrimonio utilizada por los patricios romanos era denominada *confarreatio*, en la cual la novia llevaba una

pequeña hogaza de pan, llamada *farreum*, hecha con *far* (harina de escanda menor), este ritual se celebraba ante el sacerdote de Júpiter (*flamen dialis*) y constituía una forma de matrimonio muy difícil de anular. Del término *far*, deriva la palabra *farina*, de la que procede la palabra harina y del término *adoreum* o *ador*, deriva la palabra adorar. Otro hecho importante a este respecto es que la harina de escanda se utilizaba para hacer una salsa, denominada *mola*, con la que se embadurnaban las ofrendas dirigidas a los dioses, es a partir de este ritual donde nace la palabra inmolar, que significa recubrir con mola. Todos estos rituales vienen descritos por distintos autores clásicos como Herodoto en sus libros de *Historia*, Plinio el Viejo en su *Historia Natural* o Dionisio de Halicarnaso en su *Historia Antigua de Roma*. Aunque el uso de la escanda empezó a decrecer lentamente, quedando relegado a su uso religioso, durante 300 años, éste fue el único cereal que se usaba en Roma. El cristianismo también ha usado el pan como símbolo, la comunión cristiana tiene una gran similitud con el ritual del matrimonio romano, es muy posible que ésta última naciera a partir del mismo.

A lo largo de la Edad Media, la escanda se usaba para pagar rentas (Peña-Chocarro & Zapata-Peña, 1998). En esta época la mayoría de las tierras estaban en manos de los señores que concedían terrenos, denominados *mansos*, a los campesinos y éstos debían entregar al propietario rentas tanto en especie como en dinero.

La conservación de los trigos vestidos a través de los años ha sido posible por su continuo uso por los agricultores, pero en la actualidad han quedado restringidos tan solo a unas pocas hectáreas en muy pocas localidades españolas, donde la mecanización resulta muy dificultosa debido a lo abrupto del terreno. En España, los trigos vestidos, principalmente emmer y espelta, se cultivaron hasta la primera parte del siglo XX, pero a finales de 1960 comenzó a disminuir debido a la mecanización de las labores agrícolas y a la progresiva difusión de los trigos desnudos que resultan más rentables desde el punto de vista industrial.

Según los datos de Peña-Chocarro (1996), en esa fecha, emmer era cultivado por un solo agricultor que mantenía alrededor de 250 m^2 en la parte central de Asturias, mientras que el trigo espelta era cultivado por varios

agricultores, con un total de 5 Ha en Asturias. Según Varela et al. (1999), hay tres concejos asturianos donde aún se cultiva la escanda: Quirós donde la superficie cultivada es de aproximadamente de 3,5 Ha, Lena con unas 5 Ha cultivadas y en Aller donde la superficie cultivada no llega a una Ha. El cultivo de espelta está en menor peligro que la de einkorn o emmer ya que los agricultores las cultivan para su propio consumo. Hay también un pequeño mercado a nivel local, lo cual promueve su cultivo. Einkorn se mantenía en tres provincias andaluzas: Cádiz, Córdoba y Jaén, restringido a zonas montañosas, con sistemas de cultivo tradicional y en superficies simbólicas (unas cinco hectáreas en total). En la actualidad, se desconoce si estos trigos se siguen cultivando y en qué cuantía, si bien las últimas referencias lo sitúan exclusivamente en una explotación en Zueros en Córdoba (Peña-Chocarro, 1996).

Por razones sociales, culturales o simplemente económicos los trigos vestidos son populares de nuevo. Hoy son un alimento de moda por el cual los consumidores llegan a pagar un precio más alto que por otros productos derivados del trigo común (Peña-Chocarro & Zapata-Peña 1998). En Italia, estos cultivos reciben el nombre latino de “far” o “farro”, despiertan un gran interés por los agricultores y su área de cultivo se está extendiendo rápidamente (Perrino et al., 1994).

En los últimos años se ha producido un resurgir de los trigos vestidos debido al interés que ha despertado el uso de técnicas de bajo impacto (D’Antuono, 1989), así como a la demanda de productos tradicionales, considerados ecológicos. Algunos autores han descrito que espelta y/o emmer tienen mayor contenido nutritivo (Skrabanja et al., 2001) y una digestión más fácil que la de los trigos harineros comunes. Además, espelta ha sido recomendado para el tratamiento de la colitis ulcerosa, neurodermitis y otras alergias y también para el alto contenido de colesterol en sangre (Strehlow et al., 1994; Italiano & De Pascuale, 1994). No obstante, debemos señalar que los datos científicos de las propiedades nutricionales de ambas especies son escasos y poco concluyentes. Así otros autores sugieren que la composición química de espelta no ofrece ventajas en comparación al trigo común (Ranhotra et al., 1995; Grela 1996). Su interés también se debe a su

potencial como fuente de genes para el mejoramiento de los trigos desnudos (Sharma et al., 1981; Srivastava & Damania, 1989), debido a su resistencia a varias enfermedades. Así, se ha citado resistencia a roya amarilla (causada por *Puccinia striiformis* Westend f. sp. *tritici*) (Kema, 1992), carbón común (causada por *Tilletia caries*) (Percival, 1921; Damania et al., 1990) y mildium pulverulento (causada por *Erysiphe graminis* f. sp. *tritici*) (Sjukov & Schevclenco, 1996).

En nuestro país han sido escasas las investigaciones sobre los trigos vestidos. Merece la pena destacar el trabajo de Rodríguez-Quijano et al. (1990), sobre la variabilidad de subunidades de alto peso molecular de glutenina en espelta y el de Rodríguez-Quijano et al (1997), sobre la variabilidad de las subunidades de bajo peso molecular de gluteninaen trigo einkorn, encontrándose en ambos un cierto grado de variabilidad. Recientemente, han sido analizados en nuestro grupo, la composición de las proteínas de reserva en una colección de trigo emmer español, *T. turgidum* ssp. *dicoccum*, (Pflüger et al., 2001) encontrándose un alto grado de variación para las subunidades de alto peso molecular de glutenina, ya que se detectaron trece variantes alélicas, cuatro de las cuales no habían sido descritas con anterioridad.

El objetivo de esta tesis es caracterizar la variabilidad y diversidad genética de una amplia colección de trigo espelta de origen español, mediante el uso de marcadores moleculares como son las proteínas de reserva y su posible uso como recursos genéticos en la mejora genética de la calidad del trigo.

Capítulo 2

2.1 Allelic variation of the HMW glutenin subunits in Spanish accessions of spelt wheat (*Triticum aestivum* ssp. *spelta* L. em Thell.) [Variación alélica para las subunidades de alto peso molecular de glutenina en trigo espelta de origen español]

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Resumen

Espelta (*Triticum aestivum* ssp. *spelta* L. em. Thell.) es un trigo vestido de origen iraní que sobrevive en zonas marginales de Asturias (Norte de España). La composición para las subunidades de alto peso molecular de glutenina de 403 entradas de espelta de origen español, ha sido analizada mediante SDS-PAGE. Tres variantes alélicas fueron detectadas para el locus *Glu-A1*. Para el locus *Glu-B1*, dos de los siete alelos detectados no habían sido encontrados con anterioridad; mientras cuatro de los nueve alelos detectados para el locus *Glu-D1* no habían sido descritos previamente. Considerando los tres loci, veinticinco combinaciones se encontraron a lo largo de todas las líneas evaluadas. Este amplio polimorfismo podría usarse para transferir nuevos genes de calidad al trigo, y ampliar la base genética de los mismos.

Palabras clave: diversidad genética, trigos vestidos, gluteninas.

Abstract

Spelt wheat (*Triticum aestivum* ssp. *spelta* L. em. Thell.) is hulled wheat of Iranian origin that survives at marginal areas in Asturias (Spain). The HMW glutenin subunit composition of 403 accessions of spelt wheat from Spain has been analysed by SDS-PAGE. Three allelic variants were detected for *Glu-A1*. For the *Glu-B1* locus, two of seven alleles detected have not been found before; while four of nine alleles detected for the *Glu-D1* are not previously described. Considering the three loci, twenty-five combinations were found among all the evaluated lines. This wide polymorphism could be used to transfer new quality genes to wheat, and widen the genetic basis of them.

Keywords: Genetic diversity, hulled wheat, glutenins.

Introduction

Modern agronomic practices have reduced the genetic variability of cultivated wheats, which has given great importance in the search for species that could be useful in contributing genes for wheat improvement (Jauhar, 1993). Between these species, the hulled wheat such as einkorn (2n=2x=14, **AA**; *Triticum monococcum* ssp. *monococcum* A. et D. Löve), emmer (2n=4x=28, **AABB**; *Triticum turgidum* ssp. *dicoccum* L. em. Thell.) or spelt (2n=6x=42, **AABBDD**; *Triticum aestivum* ssp. *spelta* L. em Thell.) have proved to be rich-sources of useful genes (Srivastava and Damania, 1989). Unfortunately, because of the progressive neglect of these crops by other more-economic profit, most of the genetic resources for these species are only present in germplasm banks. The increasing demand for unconventional foods, together with the search for low-input, agriculture has led to a revival of traditional food where hulled wheats could play an important role.

In Spain, the hulled wheats, mainly emmer and spelt, were widely cultivated during the first part of the 20th Century, but decreased towards the late 1960s when agricultural mechanisation in many areas of Spain began. In the case of spelt, this species survives in marginal farming areas of Asturias (North of Spain), where it is named as escanda (this term has been applied to both emmer and spelt) and is endangered (Peña-Chocarro and Zapata-Peña, 1998).

The analysis of the seed storage proteins (glutenins and gliadins) has been a useful tool for plant breeding, due to their relationship with the technological properties of wheat. Among these proteins, the best studied are the high-molecular-weight (HMW) glutenin subunits which are coded at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci on the long arm of group 1 homoeologous chromosomes in hexaploid wheat (Payne, 1987). Each locus consists of two linked genes, which code for two types of

HMW glutenin subunits with different mobility in SDS-PAGE, and named as the *x*- and *y*-type (Harberd et al., 1986).

Investigations on seed storage-protein composition have been scarce between the hulled wheats from Spain. Recently, the seed storage protein composition of a collection of Spanish emmer wheat has been analysed by our group (Pflüger et al., 2001). We believe that the high degree of variation detected could be used for widening the genetic basis of wheat. For spelt wheat, Rodriguez-Quijano et al, (1990) analysed a collection of 118 accessions, which are part of the material used in the present work, and found a low variability for the HMW glutenin subunits.

The main goal of the present study was to analyse the HMW glutenin subunit composition of an extensive collection of spelt accessions collected in the North of Spain at the first half of 20th Century.

Material and methods

Four hundred and three accessions of spelt wheat, obtained from the National Small Grain Collections (Aberdeen, USA) and Centro Nacional de Recursos Fitogenéticos INIA (Alcalá de Henares, Spain), were analysed in this study.

Proteins were extracted from crushed endosperm. Before glutenin solubilisation, the gliadins were extracted with a 1.5 M dimethylformamide aqueous solution following a double-wash with 50% (v/v) propan-1-ol at 60ºC for 30 min with agitation every 10 min. Glutenin was solubilised with 250 µl of buffer containing 50% (v/v) propan-1-ol, 80 mM of Tris-HCl pH 8.5, and 2% (w/v) dithiothreitol at 60ºC for 30 min. After centrifugation, 200 µl of the supernatant were transferred to a new tube, mixed with 3 µl of 4-vinylpyridine, and incubated for 30 min at 60ºC. The samples were precipitated with 1 ml of cold-acetone. The dried pellet was solubilised in buffer containing 625 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, and 2% (w/v) dithiothreitol in a 1:5 ratio (mg/µl) to wholemeal.

Reduced and alkylated proteins were fractionated by electrophoresis in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH 6.8/8.8) at an 8% plyacrylamide concentration (w/v, C=1.28%) with and without 4 M urea. The Tris-HCl/glycine buffer system of Laemmli (1970) was used. Electrophoresis was performed at a constant current of 30 mA/gel at 18ºC for 30 min after the tracking dye migrated off the gel. 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.

Results and Discussion

One of the principal problems of the germplasm bank is that many accessions have not been swon for many years, due to the scarce interest that the same species had in the second half of the 20th Century. This could condition their seedling production and the future of these collections. Between these species stands of the hulled wheats (einkorn, emmer and spelt) have been neglected in the greater part of their traditional land area. Because of in the increased interest for these species in recent years, we have iniciated a programme of evaluation of the variability of hulled Spanish wheats for regaining, mainly, the lines with an interest for quality.

In the Figure 1, the HMW glutenin subunit composition of several spelt accessions representative of variation detected is shown. Up to 19 allelic variants (three alleles at the *Glu-A1* locus, seven at the *Glu-B1* locus, and nine at the *Glu-D1* locus) were found in the evaluated accessions (Table 1).

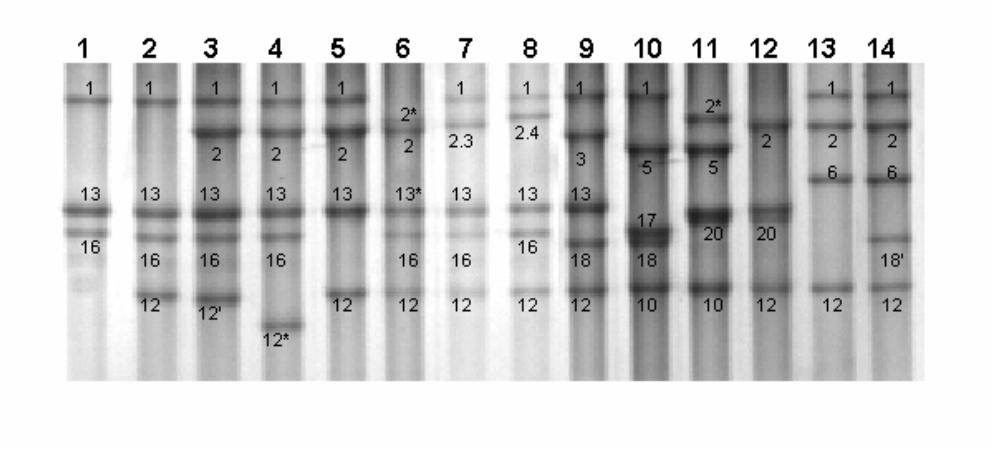

Figure 1.- SDS-PAGE (8%) patterns of HMW-Gs from some Spanish accessions of spelt wheat, representative of the different allelic variants detected at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci. Lanes: 1 Alaga, 2 PI-348680, 3 PI-348495, 4 PI-348672, 5 PI-348676, 6 PI-348767, 7 PI-348465, 8 PI-348473, 9 PI-348752, 10 Yecora, 11 BG-020900, 12 PI-190963, 13 PI-591900, and 14 PI-190960

These results showed a sharp discrepancy with the findings of Rodriguez-Quijano et al. (1990), who reported only three allelic variants at the *Glu-B1* locus and four at the *Glu-D1* locus. Six novel variants were identified by us, two at the *Glu-B1* locus and four at the *Glu-D1* locus. These new alleles were named according the progressive Roman numeral nomenclature of Vallega and Waines, (1987) and Branlard et al. (1989), which we used for the classified new allelic variants in emmer wheat (Pflüger et al., 2001). The International nomenclature indicated by McIntosh et al. (1998) has been employed to name the alleles previously described.

Similar to other cultivated wheats (Shewry et al., 1992), only one active component was found for the *Glu-A1* locus. All three allelic variants detected were previously described in spelt wheat by Rodriguez-Quijano et al. (1990); however, the frequencies of the subunits 2* and *null* were different from those found by these authors. In our material, the *null* allele (*Glu-A1c*) appeared in only four accessions (PI-190963, PI-348626, PI-348750 and BG-002014), whereas it was found in 15

accessions by Rodriguez-Quijano et al. (1990). The subunit 2* coded by the *Glu-A1b* allele (Figure 1, lanes 6 and 12) appeared in 51 accessions, which is clearly different from the findings of Rodriguez-Quijano et al. (1990) where this subunit was present in only three accessions. In both studies, the most frequent allele was *Glu-A1a* (subunit 1) which was found in 348 of the 403 accessions evaluated in the present work.

Table 1.- Allelic frequencies at the three glutenin loci in 403 Spanish spelt wheat landraces

Locus	Allele[a]	HMWGs	Accessions		Previous work[b]	
			No.	%	No.	%
Glu-A1	a	1	349	86.60	100	84.70
	b	2*	50	12.41	3	2.60
	c	Null	4	0.99	15	12.70
Glu-B1	e	20	5	1.24	-	-
	f	13+16	354	87.84	103	87.30
	an	6	1	0.25	-	-
	at	13+18	23	5.71	14	11.90
	as	13	4	0.99	1	0.80
	XVIII	13*+16	13	3.23	-	-
	XIX	6+18'	3	0.74	-	-
Glu-D1	a	2+12	359	89.08	106	89.90
	b	3+12	35	8.68	4	3.30
	d	5+10	1	0.25	-	-
	r	2.3+12	2	0.50	3	2.60
	1	*null*+12	2	0.50	5	4.20
	I	2+12'	1	0.25	-	-
	II	2+12*	1	0.25	-	-
	III	2.4+12	1	0.25	-	-
	IV	2.5+12	1	0.25	-	-

[a] According to McIntosh et al. (1998). The new alleles appear indicated with a Roman numberal.
[b] Rodriguez-Quijano et al. (1990).

For the *Glu-B1* locus, the allele *Glu-B1f* (subunits 13+16) was the most frequent among the evaluated lines of spelt wheat (87.59%). By contrast, this allele, associated with good bread-quality, is rare or appears with a low frequency in bread wheat (Payne and Lawerence, 1983; Branlard and Le Blanc, 1985; Lawrence, 1986; Lukow et al., 1989). The other two alleles previously described by Rodriguez-Quijano et al. (1990) were found in four accessions for the *Glu-B1as* allele (subunits 13+18; Figure 1, lane 10). Furthermore, five alleles which were not found by Rodriguez-Quijano et al. (1990) were detected in our material. Some of these

alleles have been described in durum and bread wheat, while other ones have not been previously found. In the latter cases, we have named the subunits according to their proximity to the subunits previously described. For example, subunit 13* that appears with the subunit 16 (Figure 1,lane 6) was slightly faster than subunit 13. This novel allele (*Glu-B1-XVIII*) was found in 13 accessions.

The allele *Glu-B1an* (subunit 6) was rare and was only found in the PI-591900 accession (Figure 1, lane 13); it was detected in two landraces of bread wheat by Rodriguez-Quijano et al. (1990) but not in spelt wheat. Other allele did not detected in spelt wheat, the allele *Glu-B1e* (subunit 20; Fig. 1, lanes 11 and 12) was found in five accessions (Table 1). The new allele *Glu-B1-XIX* present two major components, subunit *x* with a mobility similar to subunit 6, and subunit *y* similar to subunit 18 (Figure 1, lane 14), which was found in only one accession (PI-190960).

The lines evaluated present a large homogeneity for the *Glu-D1* locus, in fact 89.08% of them showed the *Glu-D1a* allele (subunits 2+12). Although these subunits have been associated with poor quality in bread wheat, it is important to emphasize that the species evaluated here is not bread wheat and, consequently, the characteristics of the different alleles detected may not be similar to bread wheat. Another allele found with a relatively high frequency was *Glu-D1b* (subunits 3+12; Figure 1, lane 9), which appears in 35 accessions. The rest of the alleles found appear in one or two accessions (Table 1). This is the case for subunits 5+10 (allele *Glu-D1d*), associated with good quality in bread wheat, and which appear in only one accession (BG-020900). The subunit 12' (PI-348495) and 12* (PI-348672), that appear with subunit 2 (alleles *Glu-D1-I* and *Glu-D1-II*, respectively), showed differences in mobility with respect to subunit 12. Subunit 12' was slightly faster than subunit 12, while subunit 12* showed a clear difference to both subunits (Figure 1, lanes 3, 4 and 5, respectively). Another novel allele, named *Glu-D1-III* (subunits 2.4+12), was detected in only one accession (PI-348473). Subunit 2.4 was slower than subunit 2.3 (Figure 1, lanes 8 and 7, respectively). This last allele (*Glu-D1r*, subunits 2.3+12) was detected in two accessions (PI-348465 and PI-348720), like the allele *Glu-D1l*, subunit 12 (PI-348680 and PI-348778).

Our previous investigations carried out with other species, such as *Hordeum chilense* Roem. et Schult. (Alvarez et al., 2001) and emmer wheat (Pflüger et al., 2001), have shown that the variability detected by normal SDS-PAGE gels could be lower than real. This seems to be due to conformational differences between the proteins (Goldsbrough et al., 1989), which cause the anomalous mobility of some subunits that appear in similar positions to the other subunits described. This is eliminated by addition of a strong denaturant, such as 4 M urea (Goldsbrough et al., 1989; Lafiandra et al., 1993) used in the present work.

When urea was added to the gel, the mobility of all subunits showed changes which differentiate some subunits better. For example, subunit 2* appears clearly separate from subunit 2, which is not always possible in normal SDS-PAGE gels (Figure 2, lanes 3 and 5). Another subunit that showed more clear differences than in normal gels was subunit 18 present in the alleles *Glu-B1at* (13+18) and *Glu-B1-XIX* (6+18). These subunits have similar mobility in normal gels (Figure 2A, lanes 3 and 5), while in the urea gel, subunit 18 of allele *Glu-B1-XIX* was faster than that of the allele *Glu-B1at* (Figure 2B, lanes 3 and 5). Consequently, subunit *y* of the first allele has been named as 18'. Furthermore, the position of some subunits in urea-SDS-PAGE gels suggested the presence of new alleles. The allele present in the PI 348572 accession that was catalogued as *Glu-D1-III* (2.4+12) showed a different mobility to the component *x* in the urea gel. This new subunit, named 2.5, is slightly slower that subunit 2.4 (Figure 2, lanes 1 and 2, respectively). This new allele formed by subunits 2.5 and 12 was named *Glu-D1-IV*.

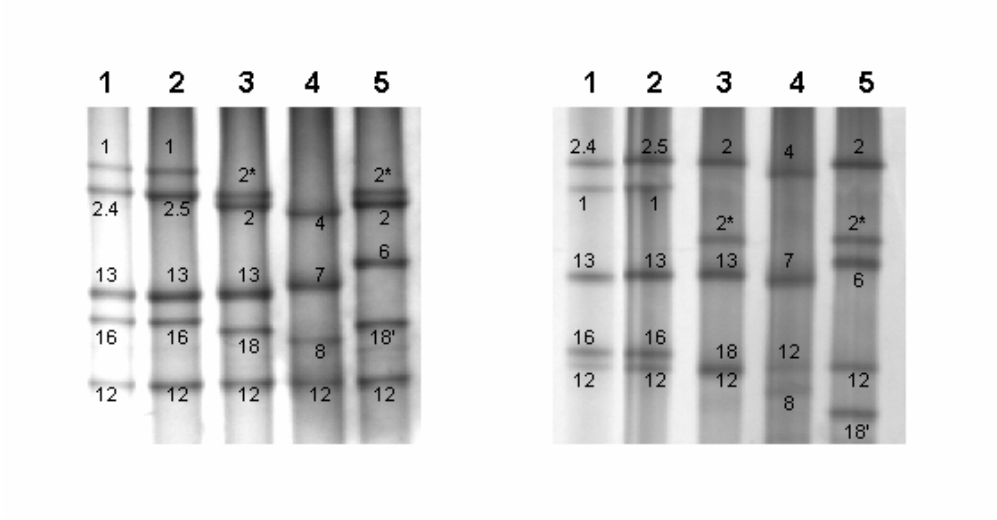

Figure 2.- SDS-PAGE without (**A**) and with (**B**) 4M urea of some allelic variants detected at the *Glu-B1* and *Glu-D1* loci. Lanes: 1 PI-348473, 2 PI-348572, 3 PI-348627, 4 Champlein, and 5 PI-348631.

For the *Glu-A1*, *Glu-B1* and *Glu-D1* loci, 25 combinations were detected. Their frequencies are shown in Table 2, where there is a clear dominance of the combination 1, 13+16, 2+12 that appears in 67.74% of the accessions. This was also the most frequent combination in the study of Rodriguez-Quijano et al. (1990), where it appeared in 75 of the 118 accessions evaluated. Other combinations found for these authors as null, 13+16, 2+12 and 1, 13+18, 2+12 showed a sharp difference with our results (Table 2). The first combination was found in only two accessions, whereas 13 accessions were identified by Rodriguez-Quijano et al. (1990). This notable difference is related with the scarce presence of the *null* allele (*Glu-A1c*) in our material as opposed to those analysed by these authors. The rest may be made up of two combinations, which appeared with a relatively high frequency; the combination 1, 13+16, 3+12 appear in 27 accessions while the 2*, 13+16, 2+12 combinatory was found in 41. Eleven of the combinations appeared in only one accession. Between them these make up the 2*, 20, 5+10 (BG-020900) combination, which shows good alleles for the *Glu-A1* and *Glu-D1* loci according to

the indicated in bread wheat. By contrast, nine combinations present the six novel alleles, whose effects on bread making quality have not been evaluated at yet.

Table 2.- Frequencies of the HMW glutenin subunits compositions found among 403 accessions analysed.

Subunit composition			No.	%	Accession standards
Glu-A1	Glu-B1	Glu-D1			
null	20	2+12	2	0.50	-
null	13+16	2+12	2	0.50	-
1	13	2+12	4	0.99	-
1	20	2+12	1	0.25	PI-348687
1	6	2+12	1	0.25	PI-591900
1	6+18’	2+12	2	0.50	-
1	13+16	12	2	0.50	-
1	13*+16	2+12	8	1.99	-
1	13+16	2+12’	1	0.25	PI-348495
1	13+16	2+12*	1	0.25	PI-348672
1	13+16	2+12	273	67.74	-
1	13*+16	3+12	3	0.74	-
1	13+16	3+12	27	6.70	-
1	13+16	2.3+12	2	0.50	-
1	13+16	2.4+12	1	0.25	PI-348473
1	13+16	2.5+12	1	0.25	PI-348572
1	13+18	2+12	21	5.21	-
1	13+18	3+12	1	0.25	PI-348752
2*	20	2+12	1	0.25	PI-469037
2*	20	5+10	1	0.25	BG-020900
2*	6+18’	2+12	1	0.25	PI-348631
2*	13*+16	2+12	2	0.50	-
2*	13+16	2+12	40	9.93	-
2*	13+16	3+12	4	0.99	-
2*	13+18	2+12	1	0.25	PI-348627

Conclusions

The variability detected in the present work showed sensitive differences with the finding of Rodriguez-Quijano et al. (1990), who used part of the present material. The cause of these differences could be due to two facts, the different number of accessions analysed (118 of the 403) and the use of different types of gel. In fact, we have used a low-polyacrylamide-concentration gel that permits best

discrimination at the level of the HMW glutenin subunits. Consequently, certain variants present here might have been overlooked by these authors and classified with a different number. This variability has been confirmed with the used of the urea-SDS-PAGE gel, which showed the presence of some subunits more clearly than did the normal SDS-PAGE gel.

Diverse studies carried out on the etnobotanical aspects of the hulled wheats (Peña-Chocarro, 1996; Peña-Chocarro and Zapata-Peña, 1998), have shown that the land area for spelt wheat has been reduced to Asturias (North of Spain) during the 20th Century. This could be cause for the presence of the combination 1, 13+16, 2+12 between the lines evaluated, which were mostly collected in this zone by personnel of the Swiss Federal Research Station for Agroecology and Agriculture during 1939 (Dr. F. Weilenmann, personal communication).

On the other hand, besides new alleles not previously described, the information may also be of interest to plant breeders for choosing parents to obtain recombinant lines with good bread-making quality. The wide polymorphism detected should be evaluated for its effects on technological properties through the transfer to bread wheat of new allelic variants or the analysis of spelt wheat itself. Consequently, we think that this species could be used as a source of genes for quality improvement in bread wheat, independent of the development of spelt as a crop in modern agriculture, which demands new products.

Acknowledgements

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2.2 Intra- and interpopulation diversity for HMW glutenin subunits in Spanish spelt wheat [Diversidad dentro y entre poblaciones para las subunidades de alto peso molecular de glutenina en trigo espelta español]

Publicado como:

L. Caballero, L.M. Martín and J.B. Álvarez. Intra- and interpopulation diversity for HMW glutenin subunits in Spanish of spelt wheat. *Genetic Resources and Crop Evolution* (in press).

Resumen

La diversidad de las subunidades de alto peso molecular de glutenina en espelta (HMWGs), *Triticum aestivum* ssp. *spelta*, fue estudiada electroforéticamente en 333 entradas agrupadas en 50 poblaciones recolectadas en Asturias, Norte de España, en 1939. La distribución dentro y entre poblaciones de las HMWGs de los loci *Glu-A1*, *Glu-B1* y *Glu-D1* fueron analizados. Los resultados mostraron que la variación genética para las HMWGs está principalmente dentro de poblaciones, siendo la variación entre poblaciones sólo del 21%. Los materiales analizados mostraron un amplio polimorfismo para las HMWGs, si bien algunas variantes alélicas fueron predominantes. Esto sugiere la posibilidad de una pérdida de variabilidad previa a la recolección, la cual podría haberse incrementado con la disminución del área de cultivo de esta especie en esta región española.

Palabras clave: variación alélica, gluteninas, trigos vestidos, mejora del trigo.

Abstract

The diversity of HMW glutenin subunits in spelt wheat, *Triticum aestivum* ssp. *spelta*, was studied electrophoretically in 333 accessions grouped in 50 populations originally collected from Asturias, North of Spain, in 1939. The inter- and intra-population distribution of HMW glutenin alleles at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci were investigated. The results show that the genetic variation in HMW glutenin subunits is mainly present within populations, being the variation between populations only 21%. The materials analysed showed a wide polymorphism for the HMW glutenin subunits, although some variants were clearly dominant. This suggests the possibility of a loss of variability before the collection that could have increased with the subsequent reduction of the cultivation area of this species in this Spanish region.

Keywords: Allelic variation, glutenins, hulled wheats, wheat breeding.

Introduction

The analysis of the seed storage proteins (glutenins and gliadins) has been a useful tool for plant breeding, due to their relationship with the technological properties of wheat. The importance of these genes in selection is based on their effects on quality wheat. Among these proteins, the best studied are the high molecular weight (HMW) glutenin subunits coded at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci located on the long arm of group-1 homoeologous chromosomes in hexaploid wheat (Payne, 1987). Each locus consists of two linked genes that code for two types of HMW glutenin subunits, with different mobility in SDS-PAGE, named *x*- and *y*-type (Harberd et al., 1986).

Past studies on these proteins have suggested that their analysis could provide a measure of genetic diversity within and between populations (Nevo and Payne, 1987). The great variability of these proteins could be consequence of the neutral selection of their cooling genes at evolutionary level. Nevertheless, the improved material showed a narrowing genetic base for these genes, which is consequence of the genetic drift occurred the genetic improvement process (Gepts, 1990). The loss of genetic diversity has promoted the search for new sources of variation that could be used in plant improvement programmes. Therefore, the collection, conservation and use of both landraces and wild relatives of cultivated species have been identified as a useful tool in the breeding programmes (Brown et al., 1989). Between these species are the hulled wheats such as spelt wheat (2n=6x=42; **AABBDD**; *T. aestivum* ssp. *spelta* L. em Thell.), which could be rich sources of useful genes (Srivastava and Damania, 1989).

In Spain, spelt wheat was grown in wide areas of the country before 20th Century, however, since 1930s, its cultivated area has progressively diminished, and

now only survives in marginal farming areas of Asturias (North of Spain), where it is endangered (Peña-Chocarro and Zapata-Peña, 1998). Due to the scarce interest for hulled wheats as spelt wheat during the last century, most germplasm of these species is stored in Germplasm Banks. Unfortunately, this material is only a snapshot of the genetic variability condemned to be slowly last. Therefore, its use in a new agriculture that searches for the new products, could be a good opportunity for the recovery of these genetic resources, before the loss of their seed germinating power would transform in fossil collections these species related to the Agriculture origins (see Padulosi et al., 1996 for review).

Studies on storage protein variability in spelt wheat from Spain have been scarce (Rodriguez-Quijano et al., 1990). Recently, an important collection (403 accessions) of these species of Spanish origin has been analysed by our group (Caballero et al., 2001). In this material, up to 19 allelic variants were found, three alleles were detected for the *Glu-A1* locus, seven alleles for the *Glu-B1* locus and nine for the *Glu-D1* locus. Some allelic variants found in this work had not been described previously. An important part of this collection (82.6%) was collected in Asturias (North of Spain) during 1939 by personnel of the Swiss Federal Research Station for Agroecology and Agriculture. These accessions were grouped by this personnel into 50 collected sites or locations by nearness criteria, they appear distributed across the geographical area where spelt was grown at that time as one population.

In this new study, we have investigated the inter- and intra-population distribution of the seed storage protein alleles at the *Glu-1* loci in these populations.

Material and Methods

Plant material

Seeds of 333 spelt wheat accessions grouped in fifty populations were analysed. These populations are distributed in the north, south and east of Asturias (North of Spain) (Figure 1). Geographical data supplied by Swiss Federal Research Station for Agroecology and Agriculture only indicated the approximated zone of collection (Dr. F. Weilenmann,

personal communication). The map of Figure 1 was drawn to show the most probable routes, according to this information and the road map of this Spanish province. These sites were both at sea level and at higher altitudes the region.

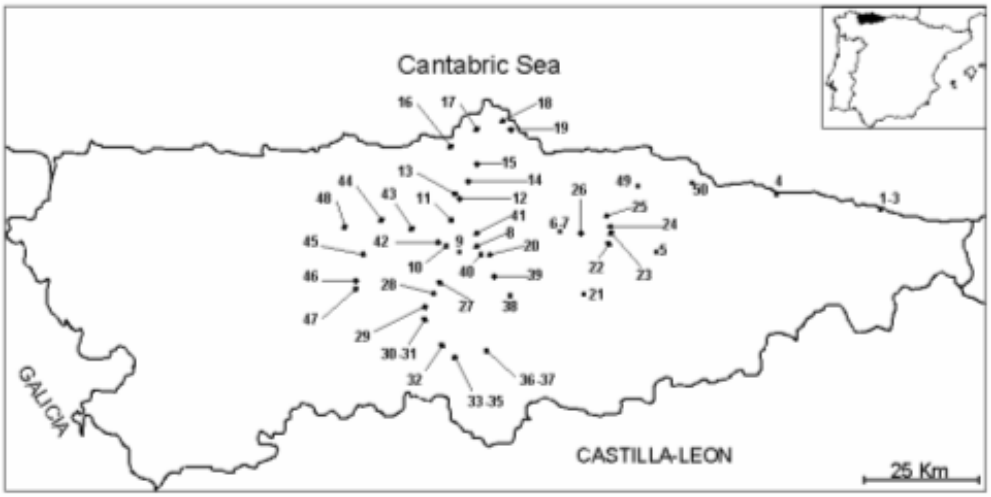

Figure 1.- Location map of the spelt populations evaluated in the present work. This material was originally collected in 1939.

SDS-PAGE analysis

Proteins were extracted from crushed endosperm. Before glutenin solubilisation, the gliadins were extracted with a 1.5 M dimethylformamide aqueous solution following a double-wash with 50% (v/v) propan-1-ol at 60ºC for 30 min with agitation every 10 min. Glutenins were solubilised with 250 µl of buffer containing 50% (v/v) propan-1-ol, 80 mM of Tris-HCl pH 8.5, and 2% (w/v) dithiothreitol at 60ºC for 30 min. After centrifugation, 200 µl of the supernatant were transferred to a new tube, mixed with 3 µl of 4-vinylpyridine, and incubated for 30 min at 60ºC. The samples were precipitated with 1 ml of cold-acetone. The dried pellet was solubilised in buffer containing 625 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, and 2% (w/v) dithiothreitol in a 1:5 ratio (mg/µl) to wholemeal.

Reduced and alkylated proteins were fractionated by electrophoresis in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH 6.8/8.8) at an 8% plyacrylamide concentration (w/v, C=1.28%) with and without 4 M urea. The Tris-HCl/glycine buffer system of Laemmli (1970) was used. Electrophoresis was performed at a

constant current of 30 mA/gel at 18ºC for 30 min after the tracking dye migrated off the gel. 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.

Statistical analysis

The following genetic variability parameters were calculated in all the populations: alleles per locus (A), percentage polymorphic loci (P), effective number of alleles pre locus (Ne) and genetic diversity (He).

The gene diversity over all populations (Ht) together with average gene diversities within (Hs) and between (Dst) populations were calculated according to Nei, (1973). The relative magnitude of genic differentiation between populations, Gst, is estimated as Dst/Ht. The interpopulational gene diversity (Rst) is given as Rst= *s*Dst/(*s*-1)Hs, where *s* is the number of populations.

The genetic identify (I) values were calculated between populations (Nei, 1972). The Nei's genetic distances (D) were used to generate an unweighted pair-group clustering based on the arithmetic averages (UPGMA) phenogram (Sneath and Sokal, 1973). This was tested by co-phenetic matrix correlation during the reconstruction of a co-phenetic matrix based on tree matrix.

Results and Discussion

Glutenin gene differentiation within and between populations

Out of nineteen alleles indicated by Caballero et al. (2001), seventeen were detected among fifty populations of spelt wheat analysed in the current work (Table1). For the three loci studied, the most frequent alleles were *Glu-A1a* (subunit 1), *Glu-B1f* (subunits 13+16) and *Glu-D1a* (subunits 2+12), respectively. Up to 223 accessions of this collection shown this composition for HMW glutenin subunits. These alleles were detected in all populations evaluated, with the exception of

population 19 that lacked the *Glu-A1a* allele and population 5 that lacked the *Glu-B1f* allele.

Table 1.- Allelic frequencies at three glutenin loci in 333 Spanish spelt wheat landraces

Locus	Allele	HMWG subunits	Accession		Populations	
			N	%	N	%
Glu-A1	a	1	288	86.5	49	98.0
	b	2*	43	12.9	25	50.0
	c	*null*	2	0.6	2	4.0
Glu-B1	e	20	1	0.3	1	2.0
	f	13+16	295	88.6	49	98.0
	at	13+18	20	6.0	12	24.0
	as	13	4	1.2	3	6.0
	XVIII	13*+16	12	3.6	9	18.0
	XIX	6+18'	1	0.3	1	2.0
Glu-D1	a	2+12	297	89.2	50	100.0
	b	3+12	29	8.7	21	42.0
	r	2.3+12	2	0.6	2	4.0
	l	*Null*+12	1	0.3	1	2.0
	I	2+12'	1	0.3	1	2.0
	II	2+12*	1	0.3	1	2.0
	III	2.4+12	1	0.3	1	2.0
	IV	2.5+12	1	0.3	1	2.0

In order to assess the distribution of alleles in different populations, the classification of Marshall and Brown, (1975) was used. For the *Glu-A1* locus, only the *Glu-A1c* allele was classified as rare (frequency ≤5%), being found in only two populations (populations 31 and 47), with a distance between them of 17 Km (Figure 1 and Table 1). The subunit 20 (*Glu-B1e*) and 6+18' (*Glu-B1-XIX*) were only found in one accession and one population (populations 40 and 32, respectively), being classified as rare with a local distribution. Other rare alleles were *Glu-B1as* (subunit 13) and *Glu-B1-XVIII* (subunits 13*+16), although the first allele was distributed sporadically in three populations (2, 39 and 50) and the second one was found in up to nine populations. For the third locus (*Glu-D1*), all the detected alleles were classified as rare, with the exception of *Glu-D1a* (subunits 2+12) and *Glu-D1b* (subunits 3+12), which were present in local distribution (Figure 1 and Table 1).

A summary of the genetic data of these three glutenin loci for the 50 populations is given in Table 2, in terms of four genetic indices. The estimated

derived here for A in spelt wheat was similar to the values in wild emmer wheat (*T. turgidum* ssp. *dicoccoides* Körn *ex* Asch. & Graebner em. Thell.) from Israel (Nevo et al., 1982) and from Turkey (Nevo and Beiles, 1989) were 1.33 and 1.22, respectively. These values were also similar to those described in *Aegilops speltoides* Tausch by Hedge et al., (2000), who analysed the genetic diversity in some wild species of Triticeae. The values present here were lower than found it in wild emmer from Jordan and Turkey (Ciaffi et al., 1993) which were 3.52 and 3.56, respectively, but were higher than reported by Nevo and Beiles, (1989) who analysed the genetic diversity in 37 populations of same species in Israel and Turkey.

The value of He (in our study He = 0.16) was slightly higher than the value in *Ae. Speltoides* (Hedge et al., 2000) and very much higher than in wild emmer wheat in Israel and Turkey (Nevo and Beiles, 1989) which were 0.10 and 0.059, respectively, but was lower than the result obtained by Nevo and Payne (1987) in wild emmer wheat in Israel (He = 0.35). The mean of Wright's fixation index (Wright, 1965) was F=1.0 in all populations, because of the observed heterozygosity was 0 in all the cases. Such values imply that spelt wheat is a self-pollinated species.

Table 2.- Storage-protein diversity based on the *Glu-A1*, *Glu-B1* and *Glu-D1* loci in 50 populations of spelt wheat.

Population	Size	A	Ne	P	He
1	6	1.67	1.35	0.67	0.260
2	6	1.67	1.22	0.33	0.180
3	2	1.00	1.00	0.00	0.000
4	5	1.67	1.31	0.67	0.230
5	3	1.00	1.00	0.00	0.000
6	7	1.67	1.21	0.67	0.170
7	9	1.67	1.16	0.67	0.130
8	7	1.67	1.21	0.67	0.170
9	11	1.33	1.13	0.67	0.110
10	5	1.33	1.13	0.33	0.110
11	4	1.33	1.16	0.33	0.140
12	7	1.67	1.25	0.33	0.200
13	2	1.33	1.28	0.33	0.220
14	6	1.67	1.35	0.67	0.260
15	7	1.67	1.35	0.67	0.260
16	4	1.33	1.16	0.33	0.140
17	5	2.00	1.55	1.00	0.350
18	8	1.00	1.00	0.00	0.000
19	4	1.00	1.00	0.00	0.000
20	13	1.33	1.05	0.33	0.050
21	4	1.00	1.00	0.00	0.000
22	6	1.67	1.35	0.67	0.260
23	3	1.33	1.21	0.33	0.170
24	8	2.00	1.40	0.67	0.280
25	4	1.33	1.16	0.33	0.140
26	2	1.00	1.00	0.00	0.000
27	9	1.67	1.16	0.67	0.130
28	10	1.33	1.12	0.33	0.110
29	10	1.33	1.06	0.33	0.060
30	8	1.67	1.34	0.67	0.250
31	8	2.33	1.50	0.67	0.330
32	9	2.33	1.55	1.00	0.350
33	6	1.33	1.19	0.33	0.160
34	7	1.00	1.00	0.00	0.000
35	3	1.00	1.00	0.00	0.000
36	6	1.33	1.11	0.33	0.100
37	6	1.33	1.11	0.33	0.100
38	6	1.33	1.11	0.33	0.100
39	10	2.00	1.37	1.00	0.270
40	11	1.67	1.13	0.67	0.110
41	7	1.67	1.35	0.67	0.260
42	7	1.67	1.30	0.67	0.230

Table 2.- Storage-protein diversity based on the *Glu-A1*, *Glu-B1* and *Glu-D1* loci in 50 populations of spelt wheat.

Population	Size	A	Ne	P	He
43	11	1.67	1.28	0.67	0.210
44	9	1.67	1.29	0.67	0.220
45	6	1.33	1.11	0.33	0.100
46	5	1.00	1.00	0.00	0.000
47	10	2.33	1.57	1.00	0.360
48	6	1.67	1.39	0.67	0.280
49	6	2.33	1.79	1.00	0.440
50	9	2.00	1.26	1.00	0.200
Mean	333	1.50	1.20	0.50	0.164

A=alleles per locus. Ne=effective number of alleles. P=percentage polymorphic loci (5%). He=genetic diversity.

A wide range of polymorphic levels characterizes the HMW glutenin subunits in the 50 populations, ranging from monomorphism to high polymorphism. As indicated by the estimate of gene diversity (He or expected heterozygosity), some populations are purely monomorphic for the three loci (e.g. populations 3, 5 or 19), or for only one (e.g. *Glu-A1* in population 22; *Glu-B1* in population 9; or *Glu-D1* in population 1). While some populations displayed low levels of polymorphism (i.e. two alleles, one largely predominating, as in *Glu-A1* in population 33, in *Glu-B1* in population 28, or in *Glu-D1* in population 20), others displayed high levels of polymorphism (i.e. 3 alleles as in *Glu-A1* in population 31, in *Glu-B1* in population 32, or in *Glu-D1* in population 24). Finally, some populations displayed very high levels of polymorphism with variation at the three loci (e.g. populations 17, 32, 47 and 49 with He of 0.350, 0.350, 0.360 and 0.440, respectively).

Additional characterization of the diversity in spelt wheat for the *Glu-1* loci is presented in Table 3. The average total genetic diversity (Ht) across the three loci in 50 populations was equal to 0.215 (ranging from 0.238 for *Glu-A1* to 0.180 for *Glu-D1*). This diversity can be divided in two components, Hs and Dst (Table 3), that measured the genie diversity within and between populations (Nei, 1973). The average relative differentiation among populations was Gst = 0.210 (ranging from 0.289 for *Glu-B1* to 0.079 for *Glu-D1*). For the three loci, data showed that 79% of

the genic diversity was within populations whereas 21% was between populations, being specially significance the low Dst data for *Glu-D1* (Table 3).

Table 3.- Differentiation of glutenin diversity with and between 50 spelt wheat populations. Ht = total gene diversity; Hs = average gene diversity within populations; Dst = average gene diversity between populations; Gst = gene diversity between populations, relative to Ht; Rst = interpopulational diversity, relative to Hs.

Locus	Alleles	Ht	Hs	Dst	Gst	Rst
Glu-A1	3	0.238	0.182	0.056	0.235	0.313
Glu-B1	6	0.227	0.161	0.066	0.289	0.415
Glu-D1	8	0.180	0.166	0.014	0.079	0.087
Mean		0.215	0.170	0.045	0.210	0.272

Genetic distances

Glutenin genetic similarity coefficients (I) were calculated for paired comparisons of all 50 populations based on the normalized identity of all loci between each pair of populations (Nei, 1972). The mean value of genetic similarity was I = 0.915, which is associated with the high homogeneity detected between populations (data not shown). Nevertheless, the genetic similarity coefficients of the population 5, 17 and 19 were very different from the average (I equal to 0.579, 0.663 and 0.614, respectively). The mean of the remaining populations without these three populations was I = 0.953. The comparison between these three populations between them showed lower values, the pairs 5-17 and 17-19 present I = 0.276, while the pair 5-19 displayed the lowest similarity with I = 0.000.

The estimates of genetic similarity between populations were independent of the collecting site. Some geographically-close populations were very different in their glutenin structure (e.g. *Glu-A1* in populations 18-19, 3.6 Km apart, have not alleles in common, I = 0.594). For contrary, geographically distant populations were similar in their glutenin composition (e.g., populations 3-46, 116 Km apart, I = 1.000) or not (e.g., populations 5-19, 40 Km apart, I = 0.000).

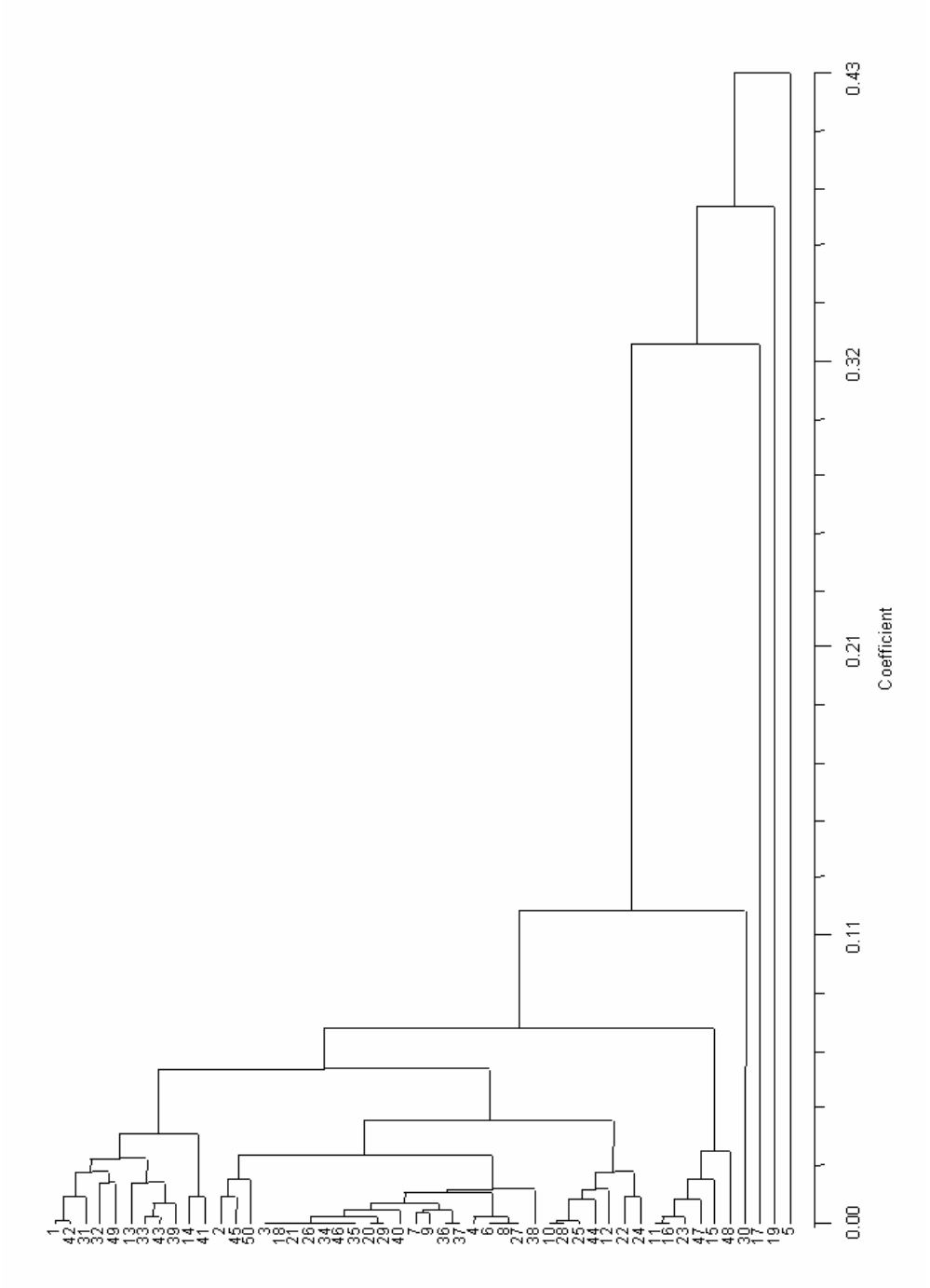

Figure 2.- Dendrogram based on Nei's genetic distance matrix between the fifty spelt wheat populations (Co-phenetic correlation = 0.910***).

Dendrogram, based on Nei's genetic distance matrix, was tested for the significance of the clustering method. A coefficient of co-phenetic correlation of r = 0.910*** was observed that represent a very good fit of a cluster to the data (Rohlf and Fisher, 1986). In the phenogram showed in Figure 2 most of the populations analysed present small genetic distance between them, being $D \leq 0.210$. Only three populations (5, 17 and 19) showed higher genetic distances (between 0.320 and 0.430), that is according to the data obtained for genetic similarity. The pattern that emerges from this dendrogram, similar to indicated for the genetic similarity, has no explanation in relation to the geographic localization of the populations analysed.

Conclusions

Data showed in the present work suggest that the seed-storage proteins of spelt wheat were not associated with adaptiveness. Their distribution could be understanding in the cultural context of agricultural society, which could have selected empirically materials with one determinated composition according to the food use. This is favoured for the fact that spelt wheat in an autogamous species that easily fixed one or other allele for each locus.

Because of the antiquity of the analysed collection (1939) and the great homogeneity within them, the loss of variability is probably a consequence of the effects of genetic drift developed often before this expedition. This loss, typical of neutral traits as are the storage proteins in cereal crops, could have increased subsequently due to the decrease in the cultivated area of this crop in this region of Spain since the end of 1960's. Consequently, the saving of these accessions, together with others stored in Germplasm Banks, is fundamental in maintaining diversity for the plant breeding of this crop, and in particular for quality improvement.

In our group, this work of regeneration has given the first results with the germination, multiplication and evaluation of the accessions with different alleles for the HMW glutenin subunits. We hope that in future, most of variability detected at the level of the other seed-storage proteins, that are being analysed, can be

recuperated. This variability could be use for the improvement of the spelt wheat *per se* or for the increase of the genetic base of the durum and bread wheats by introgression.

Acknowledgements

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Capítulo 3

3. Genetic variability for the low molecular weight glutenin subunits in spelt wheat (*Triticum aestivum* ssp. *spelta* L. em Thell). [Variabilidad genética para las subunidades de bajo peso molecular en trigo espelta (*Triticum aestivum* ssp. *spelta* L. em Thell)].

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L. Caballero, L.M. Martín and J.B. Álvarez. Genetic variability for the low molecular weight glutenin subunits in spelt wheat (*Triticum aestivum* ssp. *spelta* L. em Thell). *Theoretical and Applied Genetics* (in press).

Resumen

Se analizó la composición de las subunidades de bajo peso molecular de glutenina mediante SDS-PAGE de una colección de 403 entradas de trigo espelta (*Triticum aestivum* ssp. *spelta* L. em. Thell). Detectándose una amplia variación, 46 patrones diferentes para la zona B y 16 para la zona C. Los patrones de la zona B estaban formados desde dos a seis bandas y los de la zona C tenían de de cuatro a seis bandas en geles SDS-PAGE. Se observó un mayor número de bandas cuando se le añadió urea al gel, para la zona B los patrones mostraron de seis a once y se encontraron 14 patrones nuevos en esta zona. Para la zona C, se detectaron diez patrones nuevos que poseían de cinco a nueve bandas. Para ambas zonas, se encontraron 86 patrones diferentes. La variabilidad detectada en este material ha sido mayor que la detectada en otros trigos vestidos.

Palabras clave: trigos vestidos, trigo espelta, LMW gluteninas, SDS-PAGE.

Abstract

The low molecular weight glutenin subunits composition of a collection of 403 accessions of spelt wheat (*Triticum aestivum* ssp. *spelta* L. em. Thell) was analysed by SDS-PAGE. Extensive variation has been found, 46 different patterns for the zone B and 16 for the zone C. Patterns of the zone B exhibited from two to six bands and patterns of the zone C had from four to six bands in SDS-PAGE gels. A higher number of bands were observed when urea was added to the gels, for the zone B the patterns had from six to eleven and 14 new patterns were found in this zone. For the zone C, up to 10 new patterns that possessed from five to nine were detected. For both zones, 86 patterns were found. The variability detected in this material has been major that the detected in other hulled wheats.

Key words: hulled wheats, spelt wheat, LMW glutenins, SDS-PAGE

Introduction

Spelt wheat (2n = 6x = 42; **AABBDD**; *Triticum aestivum* ssp. *spelta* L. em Thell.) is the hexaploid hulled wheat of which would has derived the modern bread wheat (*T. aestivum* ssp. *aestivum* L. em. Thell.). This wheat would has been produced by spontaneous crossing between cultivated emmer wheat (*T. turgidum* ssp. *dicoccum* Schrank) and one wild grass (*Aegilops tauschii* ssp. *strangulata* Cross) (McFadden and Sears 1946; Kerber and Rowland 1974). This specie was widely cultivated in the past, although now is only a relict. In Spain, it is still possible to find it in marginal areas of Asturias (North of Spain) where exist a traditional agriculture with archaic practices. In our days, spelt wheat is endangered (Peña-Chocarro and Zapata-Peña 1998).

In the last years, the interest for the spelt wheat has increased, due to ecologically grown foods, the low inputs, suitable for growing without the use of pesticides, in harsh ecological conditions and in marginal areas of cultivation, and by their resistance to several diseases (D'Antuono 1989; Damania et al. 1990; Kema 1992). Furthermore, the great variation detected for the endosperm storage proteins in recent work, suggested that this specie could be used in wheat quality breeding programmes (Caballero et al. 2001; in press).

The endosperm storage proteins of wheat are divided in gliadins and glutenins. Gliadins are monomeric prolamins, controlled by the *Gli-1* and *Gli-2* loci, located on the short arms of chromosomes of the homoeologous groups 1 and 6, respectively (Payne et al. 1982; Payne 1987). Glutenins can be divided in high-molecular-weight (HMW) and low-molecular-weight (LMW) subunits. The best-studied are the HMW glutenin subunits that are coded at the *Glu-1* loci situated on the long arm of group 1 homoeologous chromosomes (Payne 1987). The genes coded for the LMW glutenin subunits, *Glu-3*, loci are located on the short arm of

these same chromosomes, and are tightly linked at the G*li-1* loci that encoding ω- and γ-gliadins (Singh and Shepherd 1988; Pogna et al. 1990).

The LMW glutenin subunits are divided into B-LMWG, C-LMWG and D-LMWG group based on their mobility in SDS-PAGE and their isoelectric points (Jackson et al. 1983). The D-LMWG subunits are the most acidic and have the lowest mobility of the LMW glutenin subunits. The C-LMWG group has a wide range of isoelectric points, and due to their mobility in SDS-PAGE overlap with α-, β- and γ-gliadins. The B-LMWG group comprises the greatest number of subunits, which are the most basic proteins, and have slightly lower mobility than α-, β- and γ-gliadins in SDS-PAGE. This group of proteins has not been studied in detail, due to their large number and considerable heterogeneity.

Biochemical and genetic aspects of wheat storage proteins have received great attention due to their importance in determining the nutritional and technological properties of cultivated wheats. Gliadins are responsible for gluten extensibility and glutenins determine gluten strength and elasticity. Although the HMW glutenin subunits have a great importance in the bread-making quality of wheat, the last studies suggest that this importance could be lower than traditionally indicated (Graybosh et al. 1990). The LMW glutenin subunits are known to be essential contributors in determining dough properties in bread wheat (Gupta and Shepherd 1988; Gupta et al. 1989; Khelifi and Branlard 1992; Zhen et al. 1997). For this reason, it may be considered the variation of the LMW glutenin subunits and gliadins and the interaction between all these components (Carrillo et al. 1990).

The main goal of this work has been the analysis of the polymorphism for the LMW glutenin subunits in a wide collection of spelt accessions of the Spanish origin.

Material and Methods

Plant material

Four hundred and three accessions of spelt wheat, obtained from the National Small Grain Collections (Aberdeen, USA) and Centro Nacional de Recursos Fitogenéticos (Alcalá de Henares, Spain), were analysed for the composition of the LMW glutenin subunits.

SDS-PAGE analysis

Proteins were extracted from crushed endosperm. Before glutenin solubilisation, the gliadins were extracted with a 1.5 M dimethylformamide aqueous solution following a double-wash with 50% (v/v) propan-1-ol at 60ºC for 30 min with agitation every 10 min. Glutenin was solubilised with 250 µl of buffer containing 50% (v/v) propan-1-ol, 80 mM Tris-HCl pH 8.5, and 2% (w/v) dithiothreitol at 60ºC for 30 min. After centrifugation, 200 µl of the supernatant were transferred to a new tube, mixed with 3 µl of 4-vinylpyridine, and incubated for 30 min at 60ºC. The samples were precipitated with 1 ml of cold-acetone and the pellet dried to air. The dried pellet was solubilised in buffer containing 625 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, and 2% (w/v) dithiothreitol in a 1:5 ratio (mg/µl) to wholemeal.

Reduced and alkylated proteins were fractionated by electrophoresis in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH 6.8/8.8) at 10% polyacrylamide concentration (w/v, C=1.28%) with and without 4M urea. The Tris-HCl/glycine buffer system of Laemmli was used (Laemmli 1970). Electrophoresis was performed at a constant current of 30 mA/gel at 18ºC for 30 min after the tracking dye migrated off the gel. 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.

Results

Some works have demonstrated that the LMW glutenin subunits are controlled by different loci (Gupta and Shepherd 1988; Ruiz and Carrillo 1993). Nevertheless, in this study, we have considered each bands group as a block, because it has been detected a great variation and the available information about the allelic segregation of them in this species is very limited. This approximation, although it is not the most correct genetically, is the most used in the variability studies.

Gels were divided in two zones, B-LMWGs and C-LMWGs, according to the used in hexaploid wheat where a clear gap was showed between these zones. A representative sample of the variation detected for both zones is shown in Figure 1.

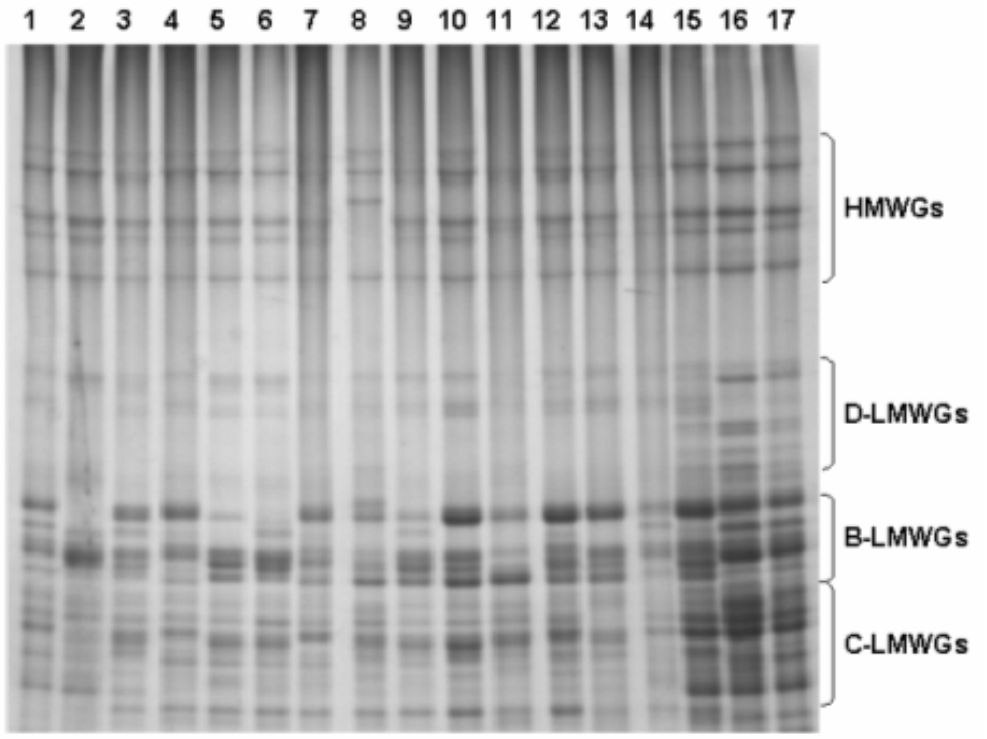

Figure 1.- Sample representative for the variation detected by SDS-PAGE of the variation found for the two zones from spelt wheat. Lanes as follow: **1**, B12, C11; **2**, B24, C12; **3**, B2, C7; **4**, B1, C2; **5**, B38, C5; **6**, B21, C5; **7**, B1, C2; **8**, B44, C7; **9**, B13, C5; **10**, B3, C7; **11**, B39, C15; **12**, B3, C7; **13**, B3, C3; **14**, B20, C1; **15**, B3, C9; **16**, B33, C8; and **17**, B27, C8

B-LMW glutenin subunits

For the B-LMWG zone, twenty-four bands were detected, which formed up to 46 different patterns. Sixteen out of these patterns were found in only one accession. Diagrams of patterns in the B-zone, with indication of their frequencies, are given in Figure 2.

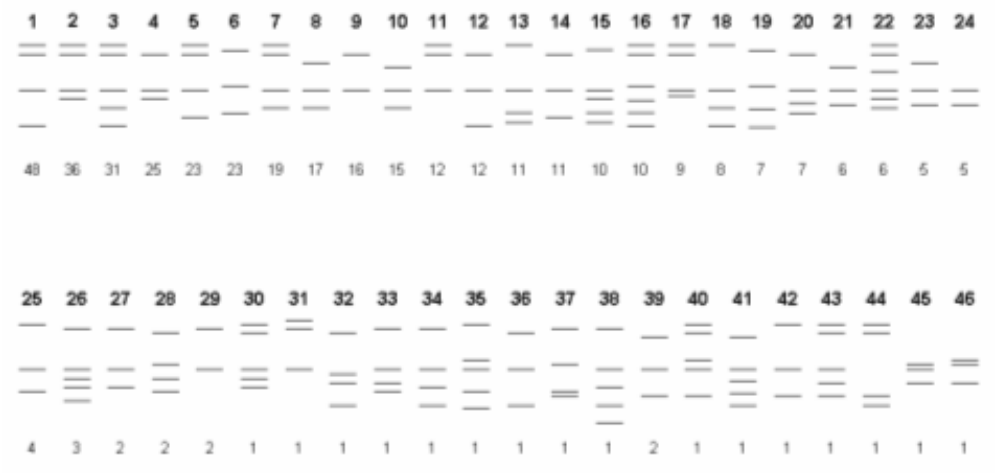

Figure 2.- Diagrammatic representation of the patterns of the B-LMWG zone. The bands with low intensity are shown with discontinuous line. The patterns are numbered from 1 to 46, in decreasing order to the presence among all accessions (first number below patterns).

The patterns were formed from two to six bands, being the most frequent the patterns with three and four bands (17 and 15 patterns, respectively). The patterns B1 and B2 were the most frequent appearing in 48 and 36 accessions, following the patterns B3 and B4 that were detected in 31 and 25 accessions, respectively. Up to 50.9% of the accessions are included in the first seven patterns (Figure 2, pattern B1 to B7), while the other 39 patterns were detected in the rest of the accessions.

Some bands of the B-LMWG zone seem to be common to several patterns; this is the case of the bands that form the pattern B9 that were observed in 15 patterns (Figure 2). Other patterns were very similar with small differences as one additional band (e.g. patterns B1 and B3 or B2 and B4) or changes in the mobility of one band (e.g. patterns B1, B5 and B7).

C-LMW glutenin subunits

For the C-LMWG zone, twenty-three different bands were detected, which formed 16 different patterns representing a lower variation than that observed in B-LMWG zone. Diagrams of these C-patterns, with their frequencies, are given in Figure 3. The patterns were formed from four to six bands. Eight out of 16 patterns only appeared in one accession. The pattern C1 was the most frequent, which was detected in 238 accessions, following of the patterns C2 and C3 that were found in 106 and 24 accessions, respectively. Considering the frequency of the patterns C1, C2 and C3 about 91.3% of all accessions belonged in these patterns (pattern C1: 59.1%; pattern C2: 26.3% and pattern C3: 6.0%).

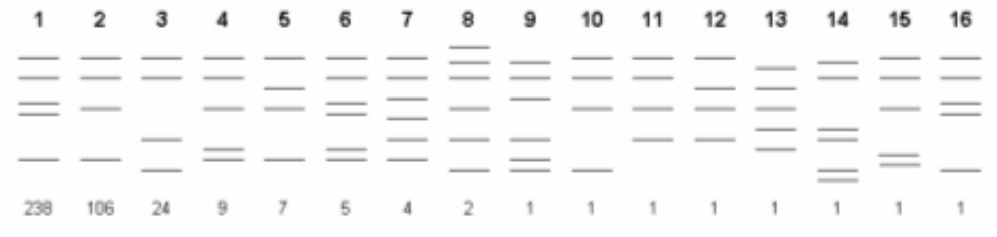

Figure 3.- Diagrammatic representation of 16 patterns of the C-LMWG zone. The patterns are numbered similar to Figure 2.

For both zones, forty-three bands were found, with a maximum of 12 bands, which formed a total of 62 different patterns. The combination of these patterns formed 100 different combinations being the most frequent the combination formed by patterns B1 and C1 (40 accessions), following of combinations B2-C2 and B3-C2 which appeared in 22 and 21 accessions, respectively. In addition, 49 combinations were found in only one accession. Generally, accessions with the same pattern for the B-LMWG zone had different pattern for the C-LMWG zone.

Characterisation of B-LMW and C-LMW glutenin subunits in SDS-PAGE with urea

Previous investigations carried out in our group have shown that the variability detected by normal SDS-PAGE could be lower than real (Alvarez et al. 2001; Caballero et al. 2001; Pflüger et al. 2001). It is possible that these proteins present anomalous mobility due to conformational differences, which cause that

some subunits appear in similar positions and they cannot be identified. This anomalous mobility is eliminated by addition of a strong denaturant, such as 4M urea (Goldsbrough et al. 1989; Lafiandra et al. 1993). In the present work, this technique has been used to differentiate some subunits.

When urea was added to gel, the mobility of all subunits showed changes and have permit to detect new patterns in B-LMWG zone, which have not been possible identified in normal gels. In these gels was found 79 different bands a number much higher than detected in normal gels, forty-four for the B-LMWG zone and thirty-five for the C-LMWG zone.

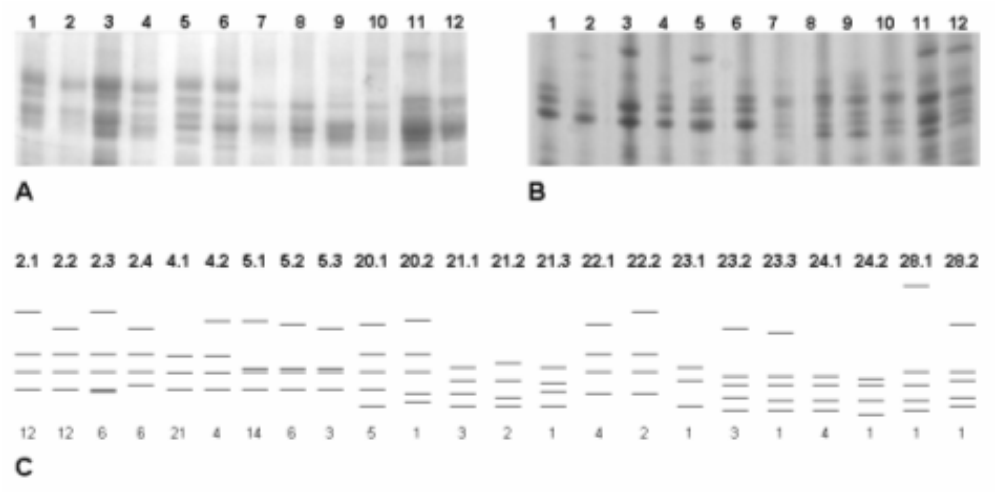

Figure 4.-. SDS-PAGE without (**A**) and with (**B**) 4M urea of same representative sample of the variation detected for the B-LMWG zone. Lanes: **1,** PI-348455, B2.1; **2,** PI-348465, B2.2; **3,** PI-348519, B2.3; **4,** PI-348572, B2.4; **5,** PI-348442, B20.1; **6,** PI-469039, B20.2; **7,** PI-348439, B21.1; **8,** PI-348478, B21.2; **9,** PI-348640, B21.3; **10,** PI-348702, B21.1; **11,** PI-348570, B23.1 and **12,** PI-348592, B23.2. **C**, Diagrammatic representation of the new B-LMWG patterns detected in 4M urea-SDS-PAGE gels. The patterns are numbered similar to Figure 2

For the zone B-LMWG zone (Figure 4), the number of bands in these the patterns varied from six to eleven bands, being the most frequents the patterns with seven bands. The change in the mobility of some bands has permits to identify up to 14 new patterns. So, the accessions PI-348455, PI-348465, PI-348519 and PI-348572 that present pattern B2 in normal gels, showed four different patterns when

urea was added to the gel (Figures 4A and 4B, lanes 1, 2, 3 and 4). The same occurred with the accessions PI-348439, PI-348478 and PI-348640 with pattern B21 in gel without urea (Figures 4A and 4B, lanes 7, 8 and 9). For the C-LMWG zone (Figure 5), the patterns were formed from five to nine bands, being patterns with six bands the most frequents and for this zone were detected 10 new patterns. For example, all the accessions presented the pattern C1 in normal gels (Figure 5A), but when urea was added to gel showed six different patterns (Figure 5B).

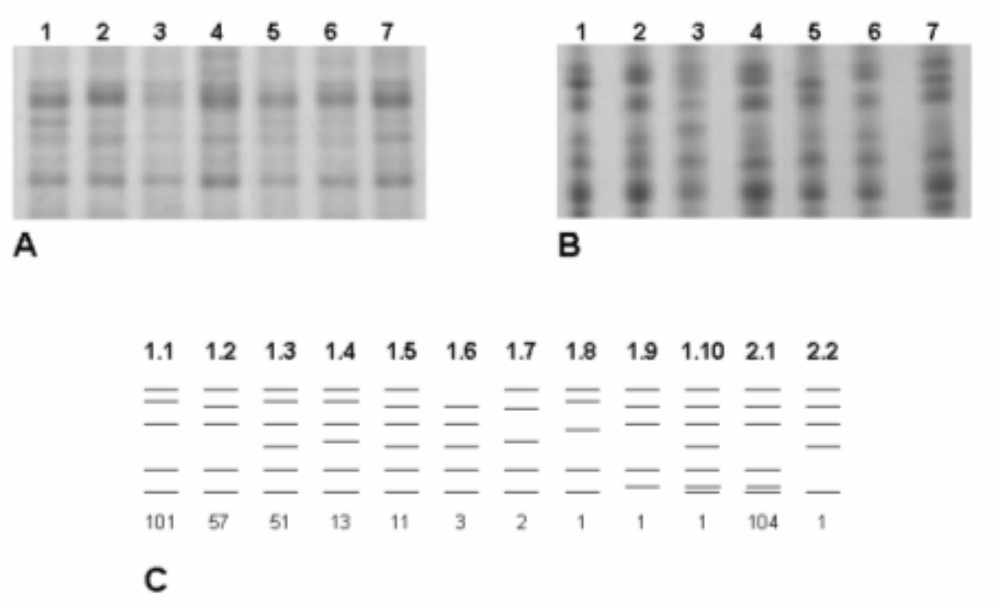

Figure 5.- SDS-PAGE without (**A**) and with (**B**) 4M urea of same representative sample of the variation detected for the C-LMWG zone. Lanes: **1,** PI-348696, C1.5; **2,** PI-348698, C1.3; **3,** PI-348702, C1.4; **4,** PI-348715, C1.1; **5,** PI-348727, C1.6; **6,** PI-348728, C1.3; and **7,** PI-348731, C1.2. **C**, Diagrammatic representation of the new C-LMWG patterns detected in 4M urea-SDS-PAGE gels. The patterns are numbered similar to Figure 2.

When both zones were analysed together, the combination formed by the patterns B8 and C13, possessed the maximum number of bands, with twenty bands, which was higher than in normal gels.

Discussion

The D-LMWG zone has not been analysed due to it is considered as contaminations of ω-gliadins (Masci et al. 1993; 1999). This zone has not been much described, although there are some works as the study of Masci et al. (1991), in which compared the B and D zones of the LMW glutenin subunits in two biotypes of the bread wheat, they found that only one of these cultivars possessed D-LMWG subunits. Also, the work of Gianibelli et al. (2002) that analysing the polymorphism of the LMW glutenin subunits in *T. tauschii* (syn. *Ae. tauschii*) identified one D-band, which had been previously described in cv. Chinese Spring by Masci et al.(1993) and Jackson et al.(1983).

The variability detected in the B-LMWG zone for spelt wheat was slightly higher than found in *T. tauschii* for the same zone by Gianibelli et al.(2002), who found thirty different patterns, and much higher than found by Nieto-Taladriz et al. (1997) in durum wheat, who detected 18 different patterns. Ruiz et al. (1998) found twenty-four different patterns in the analysis of a collection of local Spanish cultivars of durum wheat. Gupta and Shepherd (1988) detected 28 patterns for this zone when analysing 222 lines of hexaploid wheats, lower than the variability detected in spelt wheat. Some of the patterns described by these authors are very similar to the detected in our study, but we cannot affirm that they are the same because the techniques used in both studies are different.

For the C-LMWG zone, Lee et al. (1999) detected 20 different bands when studied 91 accessions of five different diploid wheat species, a number higher than detected in this work. And forty-three different patterns in this zone were observed by Gianibelli et al. (2002) in *T. tauschii*. The variation detected by these authors in diploid species was higher than the variability found by we in spelt wheat. The contrast that in this study Gianibelli et al. (2002) found more variation for the C-LMWG zone than the B-LMWG zone.

Pflüger et al. (2001) analysed the composition of the B-LMWG subunits in a collection of cultivated emmer wheat using gels with 4M urea, and they found twenty three different patterns, which are formed by two to six bands, in our study

of the variation of the LMW glutenins with 4M urea has been high, 46 different patterns for this zone which were formed from six to eleven bands. The C-LMWG zone has not been analysed in gels with urea, in these wheat species.

Unfortunately, the area where spelt wheat is still cultivated in Spain has been drastically decreased, included some zones where the materials analysed in this work were collected. Consequently, a great part of this variability could have lost in the lands. The low frequency of some patterns confirmed the necessity of protection and conservation of these accessions, because the possibility of finding the same alleles in other materials is very low. For this reason, these materials are being recuperated and multiplicated for maintaining the variability. Later, analysis on the different agronomic characters, including their quality, must be realized.

In conclusion, this study has showed that the variability for the LMW glutenin subunits in spelt wheat is higher than in other species. This variability could be used for the improvement of the spelt wheat *per se* or for the increase of the genetic base of the durum and bread wheats by introgression.

Acknowledgements

This research was supported by grant AGL2001-2419-CO2-02 from the Spanish Ministry of Science and Technology and the European Regional Development Fund from the EU. The senior author is grateful to «Ramon y Cajal» Programme of the Spanish Ministry of Science and Technology for the financial support. We thank to Bank of Germplasm INIA (CRF, Alcalá de Henares, Spain) and National Small Grain Collection (Aberdeen, USA) for supplying the spelt wheat accessions.

Capítulo 4

4. Variation and genetic diversity for gliadins in Spanish spelt wheat accessions

[Variación y diversidad genética para gliadinas en entradas de trigo espelta español]

Publicado como:

L. Caballero, L.M. Martín & J.B. Alvarez. Variation and genetic diversity for gliadins in Spanish spelt wheat accessions**.** *Genetic Resources and Crop Evolution*. (in press)

Resumen

La composición de gliadinas fue analizada en 403 entradas de trigo espelta (*Triticum aestivum* ssp. *spelta*) y se encontraron 61 patrones diferentes para las ω-gliadinas, 44 patrones para las γ-gliadinas, 19 para las β-gliadinas and 15 para las α-gliadinas. Un subgrupo de 333 de esta entradas agrupadas en 50 poblaciones procedentes de Asturias, norte de España, mostraron altos niveles de variación genética (A=3.89, P=0.88, Ne=3.350 y He=0.553), indicando que el 82.5% de la variación genética fue dentro de poblaciones, mientras que el 18.5% de esta variación fue entre poblaciones. Treinta y cinco de estas poblaciones presentaron más de cinco entradas, en este nuevo subgrupo los valores de variación genética fueron más altos que en las cincuenta poblaciones (A=4.49, P=0.91, Ne=3.80 y He=0.595). La variación genética dentro de poblaciones fue 59.7% del total y 40.3% a lo largo de las poblaciones, lo cual podría ser asociado a efectos de fijación de algunos alelos por deriva genética.

Palabras clave: diversidad genética, gliadinas, trigo espelta, *Triticum aestivum* ssp. *spelta.*

Abstract

Gliadins composition has been analysed in 403 accessions of spelt wheat (*Triticum aestivum* ssp. *spelta*); 61 different patterns were found for the ω-gliadins, 44 for the γ-gliadins, 19 for the β-gliadins and 15 for the α-gliadins. A subset of 333 accessions belonging to fifty populations from Asturias, North of Spain, showed high levels of genetic variation (A=3.89, P=0.88, Ne=3.35 and He=0.553), indicating that 82.5% of the genetic variation was within populations, and only 18.5% among populations. Thirty-five of these populations presented more of five accessions, in this new subset the values of genetic variation were higher that those of fifty populations (A=4.49, P=0.91, He=0.553; Ne=3.80 and He=0.595). The genetic variation within populations was 59.7% of the total, and 40.3% among populations, which could be associated to fixation effects of some alleles by genetic drift.

Keywords: genetic diversity, gliadins, spelt wheat, *Triticum aestivum* ssp. *spelta*.

Introduction

Genetic erosion of the common wheat germplasm genetic base caused by frequent use of the same parental genotypes for breeding activities is becoming a serious problem (Porceddu et al., 1988). During last decades, the interest for the ancient wheats has increasing, thanks to their adaptability to poor soils, harsh climatic conditions, to the low input (D'Antuono 1989), attractive nutritional attributes and demand for unconventional foods (Auricchio et al 1982; Strehlow et al 1991). In addiction, the hulled wheat -einkorn (2n=2x=14, **AA**; *Triticum monococcum* ssp. *monococcum* L.), emmer (2n=4x=28, **AABB**; *T. turgidum* ssp. *dicoccum* Schrank) y spelt (2n=6x=42, **AABBDD**; *T. aestivum* ssp. *spelta* L. Thell.) – constitute a useful gene reservoir for breeding programmes of both bread and durum wheat (see Padulosi et al. 1996 for review).

In Spain, the hulled wheats, mainly emmer and spelt, were widely cultivated during the first part of the 20th Century, to decreased towards the late 1960s, when the agricultural mechanisation started to occur in many areas of Spain. Spelt still this species survives in marginal farming areas of Asturias (North of Spain), where is endangered (Peña-Chocarro and Zapata-Peña, 1998).

Gliadins and glutenins are storage proteins of wheat endosperm. Among them gliadins, which are controlled by the *Gli* loci located on the short arms of chromosomes of the homoeologous groups 1 and 6 (Payne et al., 1982; Payne, 1987) have so far received little attention. Each *Gli* locus codes for a group of gliadin polypeptides that are inheritage as a block. Because of the multiple allelism at these loci, the different blocks generate an extremely complex gliadin pattern in hexaploid wheat (Sozinov and Poperelya, 1980; Metakovsky et al., 1984). Genes coding for most γ- and ω-gliadins have been located on the short arms of chromosomes 1A, 1B and 1D at the *Gli-A1*, *Gli-B1* and *Gli-D1* loci respectively,

whereas the genes coding for most α- and β-gliadins occur on the short arms of group 6 chromosomes at the *Gli-A2*, *Gli-B2* and *Gli-D2* (Payne, 1987).

Gliadins show the highest level of polymorphism when studied by standard method of acidic electrophoresis (Zillman and Bushuk, 1979), and have proved to be useful markers of assessing genetic variation (Lafiandra et al., 1990; Pflüger et al., 2001), and for genotype identification in different wheat species (Bushuk and Zillman, 1978; Nevo and Payne, 1987).

Investigations on seed storage protein composition have been rather frequent in bread and durum wheat; but not in hulled wheats. Seed storage proteins of a collection of Spainish emmer wheat have been analysed by Pflüger et al. (2001). Recently, both the variability for the HMW glutenins subunits (Caballero et al., 2001) and their genetic diversity (Caballero et al., in press) have been evaluated in a spelt collection collected in Northern Spain during the first half of the last century.

The main goal of the present study was to analyse the variability and the genetic diversity of gliadins in the same collection of spelt accessions.

Material and Methods

Plant material

Spelt wheat accessions (403), obtained from the National Small Grain Collections (Aberdeen, USA) and Centro de Recursos Fitogenéticos INIA (Alcalá de Henares, Spain), were analysed. Passport data on 333 of them, collected in Asturias (North of Spain) by personnel of Swiss Federal Research Station for Agroecology and Agriculture in 1939 (Dr. F. Weilessman, pers. commun.), permitted to group them in fifty populations.

Polyacrylamide gel electrophoresis (A-PAGE) analysis

Gliadins were extracted with a 1.5M dimethylformamide aqueous solution and fractionated by A-PAGE al 8.5% (C=2.67%) with low catalyst levels (ferrous sulfate and hydrogen peroxide) for increase the gel firmness (Khan et al. 1985). Electrophoresis was performed at 25mA/gel at 18ºC for 45 min after the tracking dye (methyl violet) migrated off the gel. 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.

Statistical analysis

The frequency of gliadin patterns was calculated at population and collection levels. Expected heterozygosity (He), proportion of polymorphic loci (P), average number of alleles per locus (A) and effective number of alleles per locus (Ne), were used to evaluate the genetic diversity within populations.

The gene diversity over all populations (Ht), together with the average allelic gene diversities with (Hs) and among (Dst) populations, was calculated according to Nei (1973). The relative magnitude of genetic differentiation among populations, Gst, was estimated as Dst/Ht.

Results and Discussion

Gliadins composition

Up to 72 different bands were detected, assuming that the bands with the same relative mobility represent the same subunit. These bands were grouped in to patterns at each of the four zones of gel (ω-, γ-, β- and α-gliadins); each zone was considered as a single locus and the different patterns as allelic variants. A representative sample of the variation detected is shown in Figure 1.

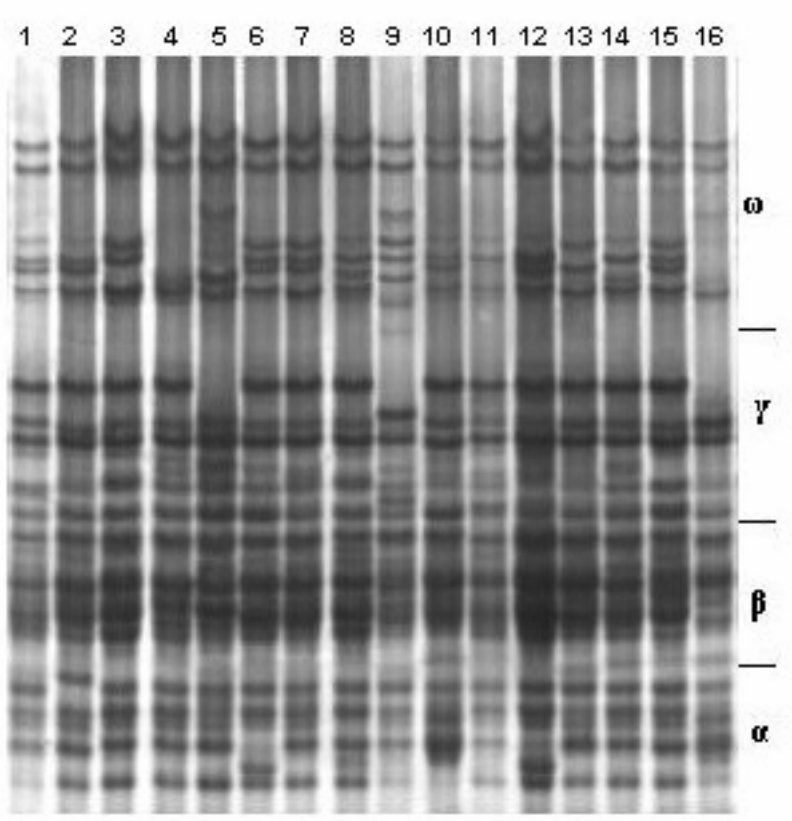

Figure 1.- Sample representative for the variation detected by A-PAGE separation of gliadins from spelt wheat.

Twenty-two different bands were found in the ω-gliadins zone, to form 61 different patterns. Diagrams of these patterns with indication of their frequencies are given in Figure 2. The number of bands present in these patterns varied from three to nine, the patterns in the ω-gliadins zone with six or seven bands being the most frequent (24.6% and 26.23 %, respectively). All patterns, except two (PI-348682 and PI-348744), had two slow bands. These two bands have been associated with the **D** genome in hexaploid wheat, but their absence does not indicate that the two accessions did not belong to the spelt groups. In fact, Caballero et al. (2001), by analysing HMW glutenin subunits of the same accessions confirmed that the genome **D** was present. Likewise, the complementary information on spike morphology confirmed that these accessions are spelt wheat.

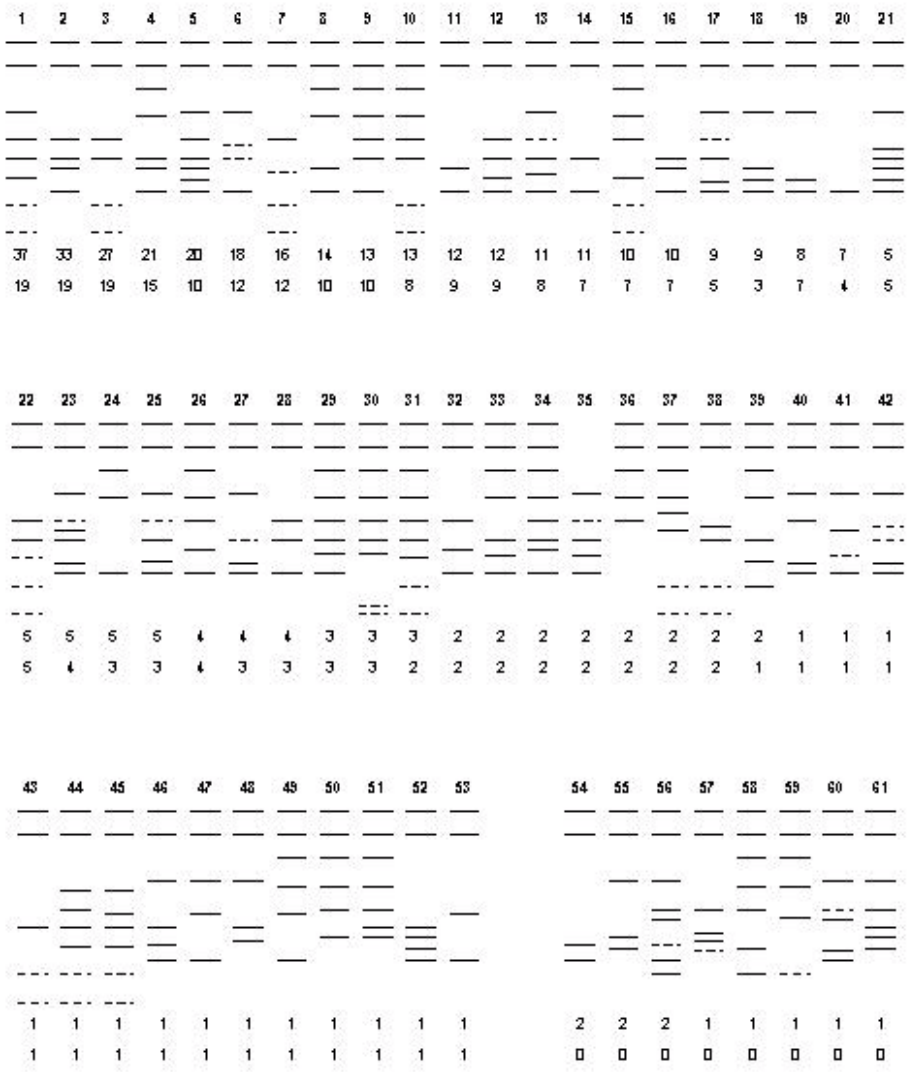

Figure 2.- Diagrammatic representation of the patterns of the T-gliadins zone. The bands with low intensity are shown with discontinuous line. The patterns are numbered from 1 to 61, in order of decreasing frequency in the accessions and the second number is the frequency in the populations.

The most frequent patterns were no. 1 and 2, which appear in 37 and 33 accessions, respectively. Other patterns with rather high frequency were 3, 4 and 5, detected in 27, 21 and 20 of the evaluated accessions. For contrary, 17 patterns were detected only in one accession.

Up to 19 bands were detected in the γ-zone, whose combination formed 44 different patterns (Figure 3). The most frequent pattern (named 1) appeared in 51 of the accessions, followed by patterns 2 and 3 that were found in 37 and 40 accessions, respectively, 16 patterns were detected only in one accession.

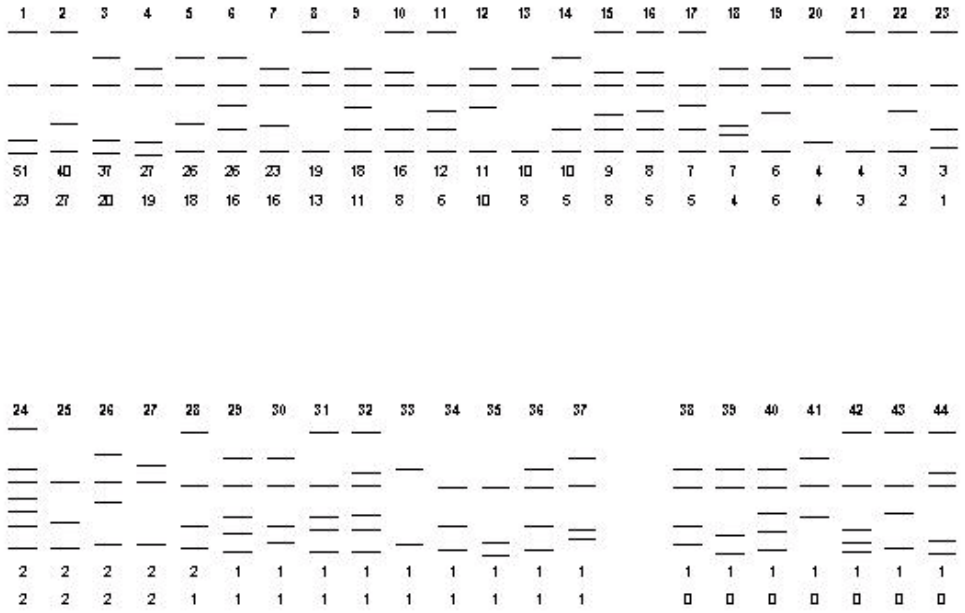

Figure 3.- Diagrammatic representation of 44 patterns of (-gliadins zone. The patterns are numbered similar to Figure 2.

Nineteen bands were detected in the β-gliadins zone, whose combination formed 19 different patterns; the most frequent the pattern of them (pattern 1) appeared in 232 accessions. Seven patterns were peculiar of 7 different accessions (Figure 4). The α-gliadins zone showed a very limited variation; with only 12 bands, whose combination formed 15 different patterns (Figure 5). Pattern 1 dominated over all others, being present in 332 out of the 403 accessions, pattern 2 was detected in only 20 accessions, whereas five patterns were present only in one accession.

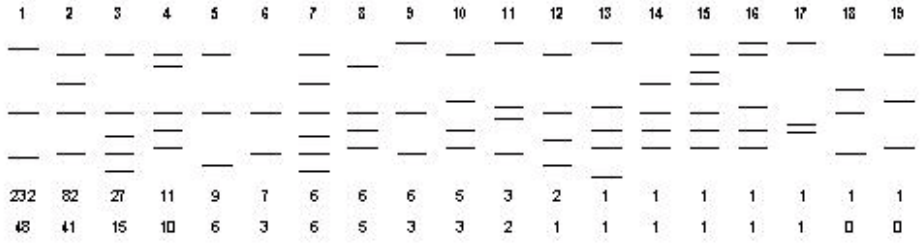

Figure 4.- Diagrammatic representation of 19 patterns of ∃-gliadins zone. The patterns are numbered similar to Figure 2.

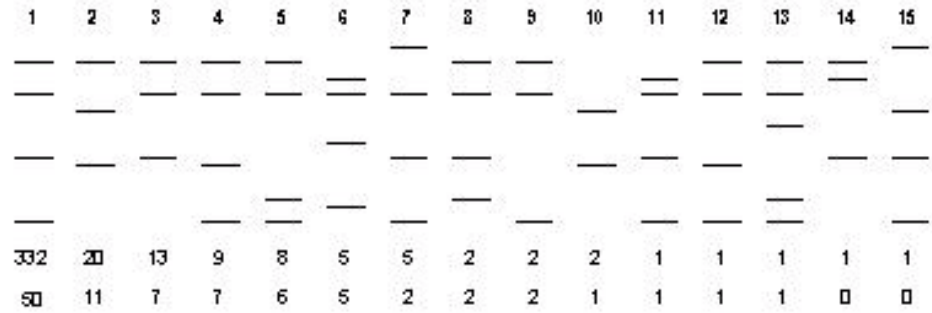

Figure 5.- Diagrammatic representation of 15 patterns of ∀-gliadins zone. The patterns are numbered similar to Figure 2.

For the four zones, some patterns derived from others by the lost of one band or successive loss of some bands (e.g. 2-11-16-20 for the ω-gliadins; 9-12-13 for the γ-gliadins, 1-2-6 for the β-gliadins and 1-3 for the α-gliadins). Others ones were consequence of the different mobility of one band (e.g. 11-14-53 for the ω-gliadins; 1-3-4 for the γ-gliadins, 1-5-9 for the β-gliadins and 1-4-5 for the α-gliadins).

Most of ω- and γ-gliadins genes are located at the loci *Gli-1*. The combination of their different patterns would give different allelic variation for this locus. In our study, were found 152 different combinations. Combination formed by patter 1 of the ω-gliadins and pattern 3 of the γ-gliadins were most frequent (32 accessions), follow of the combination of the pattern ω-5 and pattern γ-1 that appears in 19 accessions and the combination between the pattern ω-4 and γ-2 was

found in 17 accessions. On the other hand, 92 combinations appear in only one accession.

The genes coding for most α- and β-gliadins occur on the *Gli-2* loci. In this case 45 different combinations were detected, most frequents were formed by pattern 1 of the β-gliadins and pattern 1 of the α-gliadins found in 188 accessions. In 69 accessions, the combination between the pattern β-2 and α-1 was detected, while the pair β-3 and α-1 appears in 22 accessions. Nineteen combinations were only detected in one accession.

The variation found in the analysed accessions is higher than that found in other materials. Harsch et al. (1997) only found 30 different bands when analysing sixteen spelt cultivars using densitometry methods, whereas Romanova et al. (2001) detected up to 116 different patterns in 170 lines of spelt.

Distribution of variability in the evaluated populations

As previous has been described 333 out of spelt accessions evaluated in this work, were collected in 50 locations (populations) of Asturias (Northen Spain). In these accessions, a total of 53 patterns for the ω-gliadins, 37 for the γ-gliadins, 17 for the β-gliadins and 13 for the α-gliadins, were detected. The combination of these patterns supposed 128 and 39 combinations for *Gli-1* and *Gli-2* loci, respectively.

All patterns were classified following the Marshall and Brown (1975) criterium, who defined four classes of alleles based on their allele frequency and their distribution within and between populations. The alleles with frequency minor to 5% were defined as rare, in contrast to when were defined as common. Furthermore, these two classes were divided in two subclasses by geographic distribution, recognising whether the allele occurred in many populations (wide distribution) or in only one or a few adjacent populations (local distribution).

Twenty-two patterns for the ω-zone were classified as rare (frequency ≤ 5%), seventeen of them were a wide distribution appearing in most of four populations and five of them occur in only three populations each, but very distant

geographically between them (e.g. patterns 18 and 26 in populations 6-9-50 and 9-20-22, respectively). And twenty-five patterns were classified as very rare (frequency ≤1) with a local distribution.

For the γ-zone, nine common and twelve rare patterns were found, all these with a wide distribution. Additionally sixteen patterns were classified as very rare, five of them were found in only two adjacent populations (e.g. patterns 22 and 24 in populations 7-24 and 17-19, respectively) and eleven patterns were found in only one population (e.g. pattern 35 in the population 39).

All patterns of the β-gliadins were rare or very rare, except the 1, 2 and 3; appearing in most of fifteen populations. Six patterns had only local distribution. The pattern 1 of the α-gliadins was found in all populations. For these zone, four patterns were rare and four were very rare, appearing each one in only one population.

Genetic diversity

Values of genetic variation parameters are given in Table 1. The mean value of alleles per locus A was 3.89 (range from 1.50 to 6.25), much higher than those found by Hegde et al. (2000) in *Aegilops speltoides* Tausch and Caballero et al. (in press) on the same collection. These populations were very polymorphic for the four zones of gliadins, the mean value of P was 0.88, because 29 populations were polymorphic for all zones and the rest of them were polymorphic for three or two regions.

The value of He (expected heterozygosity = 0.553 in our study) was higher than that found *Ae. speltoides* by Hegde et al. (2000), by Nevo and Beiles 1989, in wild emmer wheat from Israel and Turkey (0.10 and 0.06 respectively) and Nevo and Payne (1987), which was 0.35 and higher than the same value for the same collection for the HMW glutenin subunit (Caballero et al., in press). The mean value for the effective number of alleles per locus (Ne) was 3.35 (range from 1.40 to 5.19), much higher as in the study of Caballero et al. (in press), which was 1.20.

Table 1.- Storage-protein diversity for the four zones loci in 50 populations of spelt wheat.

Population	Size	A	P	Ne	He
1	6	3.25	1.00	2.72	0.514
2	6	4.25	1.00	3.83	0.681
3	2	1.50	0.50	1.50	0.250
4	5	3.25	1.00	2.72	0.580
5	3	1.50	0.50	1.40	0.222
6	7	4.00	1.00	3.03	0.602
7	9	4.75	1.00	2.98	0.617
8	7	4.75	0.75	4.57	0.602
9	11	5.00	1.00	3.55	0.562
10	5	3.75	1.00	3.28	0.660
11	4	2.50	0.75	2.32	0.438
12	7	3.75	1.00	3.02	0.556
13	2	1.50	0.50	1.50	0.250
14	6	3.25	1.00	2.57	0.500
15	7	4.25	1.00	3.96	0.668
16	4	2.75	0.75	2.65	0.469
17	5	3.50	1.00	3.35	0.600
18	8	3.75	1.00	3.30	0.578
19	4	2.75	0.75	2.58	0.500
20	13	5.50	0.75	4.30	0.595
21	4	2.25	1.00	1.87	0.438
22	6	4.25	1.00	3.95	0.653
23	3	2.50	1.00	2.40	0.556
24	8	6.00	1.00	5.28	0.758
25	4	3.00	1.00	2.83	0.625
26	2	1.75	0.75	1.75	0.375
27	9	4.25	0.75	3.49	0.531
28	10	4.50	0.75	3.86	0.525
29	10	5.50	1.00	4.72	0.615
30	8	4.50	0.75	3.98	0.555
31	8	5.00	1.00	4.33	0.734
32	9	4.25	0.75	3.63	0.543
33	6	4.00	0.75	3.63	0.569
34	7	3.25	0.75	2.36	0.480
35	3	2.25	0.75	2.20	0.444
36	6	4.50	1.00	4.25	0.667
37	6	3.25	1.00	2.42	0.368
38	6	4.25	1.00	3.85	0.611
39	10	5.25	0.75	5.19	0.528
40	11	5.50	0.75	5.11	0.618

Table 1.- Storage-protein diversity for the four zones loci in 50 populations of spelt wheat.

Population	Size	A	P	Ne	He
41	7	3.50	0.75	3.03	0.541
42	7	3.75	1.00	2.67	0.541
43	11	6.25	1.00	4.97	0.665
44	9	5.00	1.00	4.22	0.599
45	6	3.75	0.75	3.40	0.569
46	5	2.50	0.75	2.08	0.400
47	10	5.50	1.00	4.01	0.675
48	6	5.00	1.00	4.63	0.736
49	6	4.50	1.00	4.10	0.667
50	9	5.25	1.00	4.19	0.611
Mean		3.89	0.88	3.35	0.553

Because of the small number of accessions of some populations, these parameters were also measured in the populations with more of five accessions (Table 1). Thirty-five populations were selected according to these criteria, which grouped up to 278 out of 333 accessions with passport data. Only two patterns for the □-zone (patterns 30 and 40, Figure 2) and four ones for the -zone (patterns 11, 19, 35 and 36, Figure 3) were not detected in these populations face to the complex set of Asturias populations. The data of these populations were sensitive higher than those obtained for the complex set (50 populations).

Additional characterization of the gliadin diversity for both subsets is presented in Tables 2 and 3. For the complex set of 50 populations (Table 2), the average total genic diversity (Ht) was equal to 0.703 (ranging from 0.304 for α-gliadins to 0.960 for ω-gliadins). Sensibly higher than the values obtained for HMW glutenin subunits (Ht = 0.215) by Caballero et al. (in press). The average relative differentiation among populations Gst was 18.5% (ranging from 17.0% for β-gliadins to 22.4% for α-gliadins), the genic diversity within populations was 82.4%. These values were slightly different to those obtained for HMW glutenin subunits, where the genic diversity among populations was Gst = 21.0%.

Table 2.- Differentiation of gliadin diversity within and among 50 spelt wheat populations

Zone	Patterns	Ht	Hs	Dst	Gst (%)
ω-gliadins	53	0.960	0.791	0.169	17.6
γ-gliadins	37	0.940	0.778	0.162	17.2
β-gliadins	17	0.607	0.504	0.103	17.0
α-gliadins	13	0.304	0.236	0.068	22.4
Mean		0.703	0.557	0.126	18.5

When only the populations with more of five accessions were analysed (Table 3), the data showed slightly higher values for the total genic diversity (Ht = 0.795). Nevertheless, the values within (Hs) and among (Dst) populations changed sensitively. The genic diversity among populations increased its values with a mean of 0.297. This supposed that 40.3% of total genic diversity appeared among populations, which suggest the presence of fixation effects that could produce the loss of alleles by genetic drift.

Table 3.- Differentiation of gliadin diversity within and among the 35 spelt wheat populations with more of 5 accessions/population

Zone	Patterns	Ht	Hs	Dst	Gst (%)
ω-gliadins	51	0.970	0.682	0.288	29.6
γ-gliadins	33	0.958	0.671	0.287	29.9
β-gliadins	17	0.731	0.434	0.297	40.6
α-gliadins	13	0.520	0.203	0.317	60.9
Mean		0.795	0.498	0.297	40.3

Conclusions

The data reported showed in the present work indicate that the genetic diversity for gliadins in spelt is considerably higher than that obtained for glutenins (Caballero et al., in press). This is related to the higher polymorphism detected for these proteins, which are coded for six multigenic loci located on chromosomes 1 and 6 located in of the three genomes of this species.

The very low frequency of some patterns confirmed the necessity of the protection and conservation of these accessions, because the possibility of finding the same alleles in other materials is very low. Consequently, the loss of these materials would be equivalent to the definitive loss of these alleles and the decrease of the genetic base of the wheat.

The conservation of the variability for all the endosperm storage proteins in this species could have two different applications. The quality improvement of the spelt wheat *per se* in the context of a sustainable or "ecological" agriculture, where this type of low-input crops is very appreciated. Alternatively, this variability could be used for increasing the genetic base of durum and bread wheat.

Acknowledgements

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Capítulo 5

5. Variability and genetic diversity for endosperm storage proteins in spanish spelt wheat. [Variabilidad y diversidad genética para proteínas de reserva en trigo espelta español].

Publicado como:
L. Caballero, L.M. Martín, and J.B. Alvarez. Variability and genetic diversity for endosperm storage proteins in spanish spelt wheat. In: Proceeding 8th Gluten Workshop 2003 (in press).

Resumen

Espelta (*Triticum aestivum* ssp. *spelta* L. em. Thell) es un trigo vestido hexaploide, considerado el progenitor del actual trigo harinero. La variación alélica y la diversidad genética para las gliadinas y para las subunidades de alto y bajo peso molecular de glutenina se analizó en 333 entradas españolas, que fueron recolectadas en 50 localidades de Asturias (Norte de España) hace sesenta años. En estos materiales se detectó un alto grado de variación. Al evaluar la distribución de estas variantes alélicas dentro y entre localidades se encontró que la mayor parte de la diversidad genética estaba presente dentro de localidades con un 77.3% de la diversidad genética total, siendo la variación a lo largo de las localidades de 22.7%.

Abstract

Spelt wheat (*Triticum aestivum* ssp. *spelta* L. em. Thell) is hexaploid hulled wheat that is considered the progenitor of the modern bread wheat. The allelic variation and genetic diversity for the gliadins and HMW- and LMW-glutenin subunits were analysed in 333 Spanish accessions, which were collected in 50 localities of Asturias, North of Spain, sixty years ago. A high degree of variation was detected in these materials. When the distribution among and within localities for these allelic variants were evaluated, the most of genetic diversity was present within localities with 77.3% of the total genetic diversity, being the variation among localities of 22.7%.

Introduction

The Agriculture evolution in the last fifty years has produced an erosion of the genetic base of wheats. The study of the storage proteins has showed that the frequency of some allelic variants is higher to the rest. This scarce variability in the direct consequently of the plant breeding programs, in which it has been used a limit number of sources, following of a strong press of the new cultivars obtained on the traditional varieties. Based on this, it has been made a research of new allelic in local varieties in the Germplasm Banks, between these species are the hulled wheats such as, spelt wheat (2n=6x=48; **AABBDD**; *Triticum aestivum* ssp. *spelta* L. em. Thell.). This wheat was extensively grown in Spain before 20th century, but in our days it is endangered, now only survives in marginal farming areas of Asturias (North of Spain), where traditional farming system still survive (Peña-Chocarro and Zapata-Peña, 1998).

The increasing interest in low-input system due to the actual ecological and economical situation has led to a growing interest in specific genetic variability. Sustainable Agriculture and the health food products have been gaining increasing popularity that has led to a renewed interest in hulled species such as spelt wheat (Abdel-Aal et al., 1995; Ranhotra et al., 1995). Furthermore, spelt has been described as a crop that grows on poor soils in mountain regions, tolerant to cold and excess humidity and resistant to several diseases (Damania et al., 1990; Kema, 1992). In addition, this species could be a rich sources of useful genes, such as are the endosperm storage proteins (glutenins and gliadins), which are directly relationship with the bread-making quality.

The aim of this work was the study of the genetic diversity of one wide collection of the spelt wheat from Spanish origin using the polymorphism of the endosperm storage proteins.

Material and methods

Three hundred and thirty three spelt wheat accessions from Spanish origin were analysed. Geographic distribution data supplied by Swiss Federal Research Station for Agroecology and Agriculture that allowed us grouped these accessions in fifty locations or populations.

Gliadins were extracted with 1.5M dimethylformamide and fractionated by A-PAGE al 8.5% (C= 2.67%). Reduced and alkylated glutenin subunits were separated by electrophoresis in vertical SDS-PAGE slabs in a discontinuous Tris-HCl- SDS buffer system (pH: 6.8/8.8) at an 8% and 10 % polyacrylamide concentration (w/v, C=1.28%) with and without 4M urea.

The frequency of glutenin and gliadin patterns was calculated at population and collection levels. Proportion of polymorphic loci (P), average number of alleles per locus (A) and effective number of alleles per locus (Ne), were used to evaluate the genetic diversity within populations. The gene diversity over all populations (Ht), together with the average allelic gene diversity within (Hs) and among (Dst) populations, was calculated. The relative magnitude of genetic differentiation among populations, Gst, was estimated as Dst/Ht.

Results and discussion

Variability for endosperm storage proteins

A high level of variation was found for all loci evaluated. A total of 17 allelic variants were found for the HMW glutenin subunits, three variants for the *Glu-A1* loci, six for the *Glu-B1* loci and eight for the *Glu-D1* loci, of which six had not been described previously. With all these variants it was detected 20 different

combinations, being the combination most frequent the combination formed by the variants 1,13+16, 2+12 that was found in 223 of the accessions evaluated and 19 combinations appeared only in one accession.

For the LMW glutenin subunits were detected 50 different patterns for the B-LMW group and 23 for the C-LMW group, whose combination formed 77 different combinations. The combination of the patterns B1 and C2 was the most frequent appearing in 30 accessions, following of the combination B2 and C3 that was detected in 14 accessions and 32 combinations were peculiar of 32 different accessions.

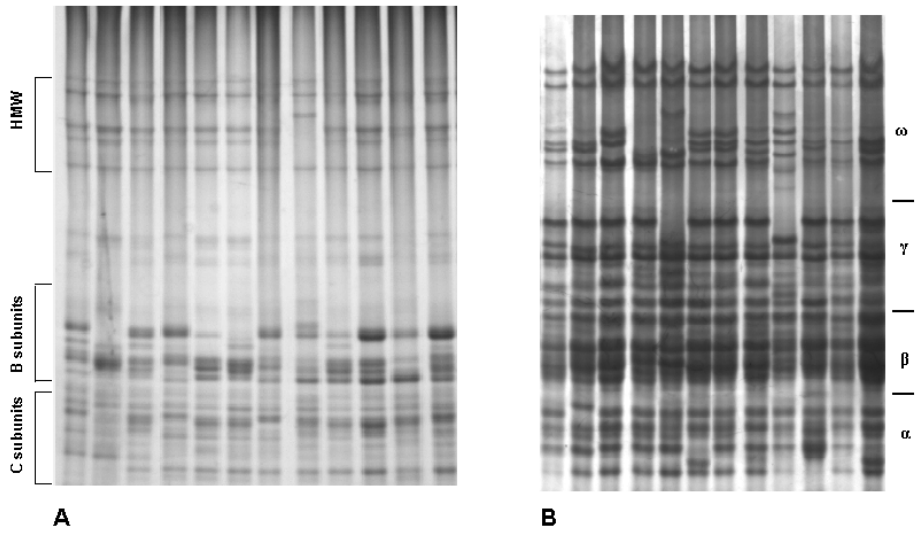

Figure 1.- Samples of variation in spelt. **A**, HMW and LMW glutenin subunits in SDS-PAGE, and **B**, gliadins in A-PAGE.

The higher level of polymorphism was found for the gliadins, 53 different patterns for the ω-gliadins, 37 for the γ-gliadins and 17 and 13 for the β- and α-gliadins, respectively. The combination of these patterns formed 128 and 39 different combinations for *Gli-1* and *Gli-2* loci, respectively, being the most frequents the combinations ω1-γ3 for the *Gli-1* loci appearing in 27 accessions and 74 combinations were unique for one accession. The combination formed by the

pattern β1 and α1 for the *Gli-2* loci was detected in 174 accessions and 13 combinations appeared in one accession.

In order to assess the distribution of variants in different populations, the criterion of Marshall and Brown was used (Marshall and Brown, 1975), who classified the variants as common when appear with frequency≥5% and rare with a frequency<5%. Furthermore, these can be of wide distribution when appear in many populations or local distribution when occur in one or a few populations. According to this classification, 38 of these variants were classified as common and wide distribution, 68 variants were rare (frequency<5%) with a wide distribution and 84 were very rare (frequency<1%) of which 54 were present in local distribution.

Genetic diversity

The mean value of A (number of alleles per locus) was 3.155 (range between 1.333 and 5.000), which was higher than this detected by Nevo and Beiles (1989), who studying 42 loci in *T. turgidum* ssp. *dicoccoides* from Israel and Turkey and also higher than this value found by Hegde et al. (2000) when they analysed the genetic diversity for 10 isozymes in 35 populations of diploid goat grass species, which were 1.50 and 1.22, respectively. The same occurred with the rest of parameters that these were higher than the last species. In these populations were detected a high level of polymorphism, being the mean value of the percentage polymorphic locus (P) was 0.76 (range: 0.33-1.00). In the last species, this value was 0.22 and 0.17, respectively.

Other characterization of the diversity in spelt for all loci is given in Table 1. The distribution among and between populations for all variants was calculated. The average of total genetic diversity, Ht, across of all loci evaluated for the fifty populations was 0.584 (range from 0.200 for *Glu-D1* loci to 0.959 for ω-gliadins).

Table 1.- Differentiation of seed storage proteins diversity within and between 50 spelt populations.

Locus	Allele	Ht	Hs	Dst	Gst
Glu-A1	3	0.237	0.166	0.071	0.300
Glu-B1	6	0.224	0.147	0.077	0.344
Glu-D1	8	0.200	0.166	0.034	0.170
B-LMW	50	0.955	0.748	0.207	0.217
C-LMW	23	0.829	0.646	0.183	0.221
ω-gliadins	52	0.959	0.755	0.204	0.213
γ-gliadins	37	0.938	0.745	0.193	0.206
β-gliadins	18	0.620	0.499	0.121	0.195
α-gliadins	12	0.294	0.230	0.064	0.218
Mean		0.584	0.455	0.128	0.231

Ht, Hs, Dst and Gst, see explain in the text

This parameter can be divided in two components, the genetic diversity within and between populations, Hs and Dst, respectively. The average relative differentiation among populations was Gst = 0.231 (range from 0.170 for *Glu-D1* to 0.344 for *Glu-B1* loci). For all loci the most genetic diversity was present with populations with 76.9% of the total genetic diversity, being the variation among populations of 23.1%. This could be associated to fixation effects of some variants by genetic drift.

Conclusions

Data showed in the present work suggest that the seed-storage proteins of spelt wheat were not associated with adaptiveness. Their distribution could understand in the cultural context of agricultural society, which could have selected empirically materials with one determinate composition according to the food use. This is favoured for the fact that spelt wheat is an autogamous species that easily fixed one or other allele for each locus.

Because of the antiquity of the analysed collection (1939) and the great homogeneity within them, the loss of variability is probably a consequence of the effects of genetic drift developed often before this expedition. This loss, typical of neutral traits as are the storage proteins in cereal crops, could have increased subsequently due to the decrease in the cultivated area of this crop in Spain since the end of 1960's. Consequently, the saving of these accessions, together with others stored in Germplasm Banks, is fundamental in maintaining diversity for the plant breeding of this crop, and in particular for quality improvement.

For this reason, these materials are being recuperated and multiplicate for maintaining the variability. Later, analysis on the different agronomic characters, including their quality, must be realized. This variability could be used for the improvement of the spelt wheat *per se* or for the increase of the genetic base of the durum and bread wheats by introgression.

Acknowledgements

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Capítulo 6

La explotación de la diversidad genética de las colecciones de germoplasma de trigo es necesaria para ampliar la base genética de este cultivo. El análisis de la variación de las proteínas de reserva en trigo es una herramienta muy útil no sólo para los estudios de diversidad, sino también para ayudar en la optimización de la variación en las colecciones de germoplasma y en la mejora de los cultivos con interés en la calidad harino-panadera.

Considerando globalmente el trabajo, se ha analizado la variabilidad existente para las subunidades de alto y bajo peso molecular de glutenina y las gliadinas. En el caso de las subunidades de alto peso molecular de gluteninas, al ser conocida la genética, se ha podido estudiar la diversidad genética analizando los alelos presentes y la frecuencia dentro y entre poblaciones de cada uno de ellos; mientras que en el caso de las subunidades de bajo peso molecular y gliadinas, dada la falta de conocimiento sobre su genética, el análisis de la diversidad genética dentro y entre poblaciones se ha basado en la consideración de patrones.

Para las tres fracciones de proteína, el nivel de variabilidad encontrado ha sido mayor que el descrito anteriormente, tanto en esta especie como en otros trigos vestidos. Gran parte de esto podría asociarse al empleo de métodos como el uso de geles SDS-PAGE con y sin urea y al empleo de distintas concentraciones, lo que permite una mejor discriminación de las distintas variantes.

En los casos en los que la genética lo ha permitido se han detectado seis alelos hasta ahora no descritos en esta especie (que han sido incorporados al Catalogue of Gene Symbols for Wheat, suplemento 2003).

En todas las fracciones evaluadas hubo unas variantes claramente dominantes sobre las demás. En el caso de las subunidades de alto peso molecular, la combinación dominante estuvo presente en 67.7% de las entradas, seguido de la combinación de los patrones β1 y α1 para el locus *Gli-2* que se detectó en el 46.7 % de las entradas; por el contrario, la mayor variabilidad para las subunidades de bajo peso molecular de glutenina y las ω/γ gliadinas hace que la combinación más frecuente para las subunidades de bajo peso molecular de glutenina esté sólo

presentes en el 9.9 % de los casos y la más frecuente para el locus *Gli-1* aparezca en el 7.9 % de las entradas.

Al cuantificar la variación y estudiar cómo se distribuye dentro y entre poblaciones para estos marcadores, se ha encontrado que la variación total oscila entre 0.200 y 0.959 y que, en promedio, el 77% está dentro de poblaciones, por lo que, en principio, parece que conservando unas pocas poblaciones se recuperaría la mayor parte de la variabilidad genética. No obstante, hay que tener en cuenta que muchos de los alelos y patrones, y en particular los alelos nuevos encontrados aparecen en una sola población, por lo que sería importante mantener la población completa y si fuese posible, incrementarla.

Así pues, del trabajo se obtienen las siguientes conclusiones:

La variabilidad encontrada para los distintos loci y patrones analizados repiten el mismo esquema: hay un gran nivel de variabilidad pero las variantes más frecuentes tienen una posición muy dominante. En todo caso, y como es lógico, este efecto es más marcado en la medida que este número es menor.

Aunque la mayor parte de la variación detectada para estos marcadores está dentro de poblaciones, las variantes raras y, en todo caso, las detectadas como novedad en trigo, están presentes sólo en una localidad, por lo que es muy importante el mantenimiento de la colección completa y sería deseable el que pudiese incrementarse.

Como consecuencia de lo anterior sería muy interesante establecer un programa de recuperación de semillas y el establecer una colección nuclear (*core collection*) que incluya las variantes detectadas y posibilite una primera evaluación en la calidad de los productos.

Todo lo anterior puede redundar en el mantenimiento "*in situ*" del cultivo de espelta, así como en su utilización en la mejora de la calidad del trigo.

Capítulo 7

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