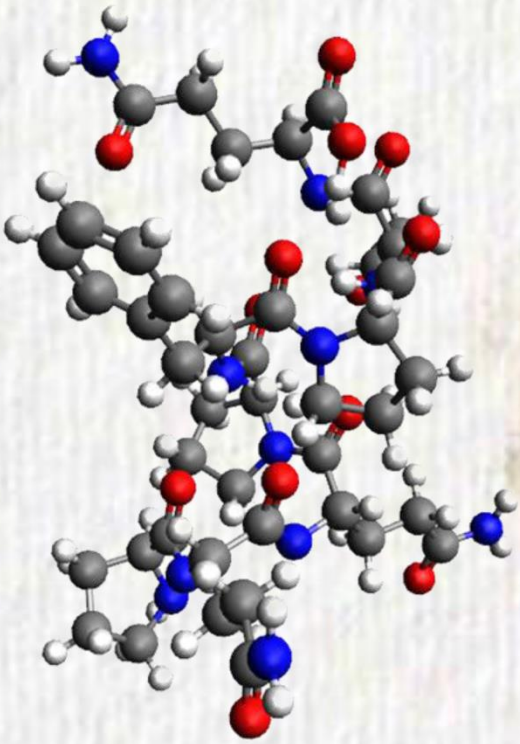
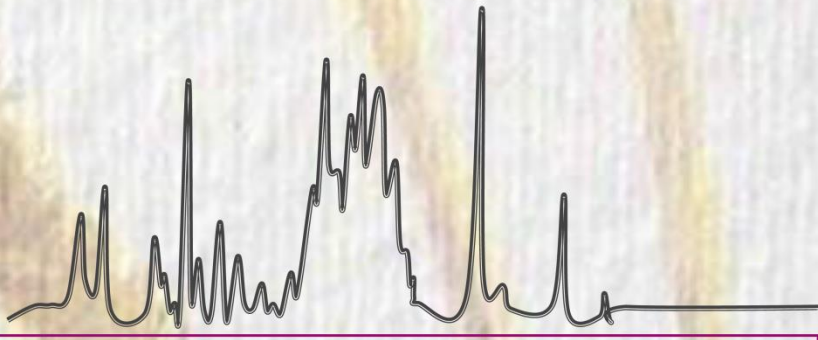
# Caracterización proteómica de líneas de trigo con muy bajo contenido en gliadinas: implicaciones para el desarrollo de alimentos aptos para el colectivo celíaco





Tesis Doctoral Mª Dolores García Molina

TITULO: *Caracterización proteómica de líneas de trigo con muy bajo contenido en gliadinas: implicaciones para el desarrollo de alimentos aptos para el colectivo celíaco*

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**UNIVERSIDAD DE CÓRDOBA**

**DEPARTAMENTO DE GENÉTICA**

Programa de doctorado

**Biociencias y ciencias agroalimentarias**

TESIS DOCTORAL

# Caracterización proteómica de líneas de trigo con muy bajo contenido en gliadinas: implicaciones para el desarrollo de alimentos aptos para el colectivo celíaco

Proteomic characterization of wheat lines with very low gliadin content: implications for the development of foods suitable for the celiac group

Autora

**Mª Dolores García Molina**

Dirigida por

**Dr. Francisco Barro Losada**

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Instituto de Agricultura Sostenible-CSIC

**Enero 2017**

**TÍTULO DE LA TESIS:** Caracterización proteómica de líneas de trigo con muy bajo contenido en gliadinas: implicaciones para el desarrollo de alimentos aptos para el colectivo celíaco

**DOCTORANDO/A:** Mª Dolores García Molina

## INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El Dr. **Francisco Barro Losada**, Investigador Científico del Instituto de Agricultura Sostenible (IAS) perteneciente al CSIC, como director de la tesis doctoral con título: "Caracterización proteómica de líneas de trigo con muy bajo contenido en gliadinas: implicaciones para el desarrollo de alimentos aptos para el colectivo celíaco", realizada por **Mª Dolores García Molina**,

Informa que:

Dicha tesis ha sido realizada bajo mi dirección.

Su principal objetivo ha sido caracterizar el proteoma de líneas de trigo con bajo contenido en gliadinas, obtenidas mediante RNAi, usando diversas técnicas de cuantificación/detección, para conocer los niveles de silenciamiento de las proteínas relacionadas con la celiaquía y determinar cómo podrían afectar estas líneas a personas que presentan intolerancia al gluten o alergia a las proteínas del trigo.

Los resultados y conclusiones obtenidos son de relevancia para avanzar en el conocimiento científico de las proteínas del gluten de trigo para el desarrollo de alimentos aptos para el colectivo celíaco.

En su etapa pre-doctoral, Mª Dolores García Molina ha realizado dos estancias de 120 días cada una de ellas, durante los años 2014 (desde abril hasta julio) y 2015 (desde marzo hasta junio) en el "Department of Agriculture, Foresty, Nature and Energy (DAFNE)" perteneciente a l'Università degli Studi della Tuscia, Viterbo (Italia) bajo la tutela de la profesora Stefania Masci. El objetivo fue el análisis proteómico de las proteínas pertenecientes (gliadinas y gluteninas) y no pertenecientes (proteínas metabólicas y solubles en cloroformo/metanol) al gluten de trigo en dos líneas que presentan las gliadinas silenciadas mediante ARNi utilizando dos promotores diferentes; ha colaborado en varias líneas de investigación y asistido a diversos congresos nacionales de los que derivan las publicaciones siguientes:

Publicaciones derivadas de la tesis:

**María Dolores García-Molina**, Francisco Barro. Characterization of changes in gluten proteins in Low-Gliadin transgenic wheat lines in response to application of different nitrogen regimes. *Frontiers in Plant Science (*under review).

**María Dolores García-Molina**, Vera Muccilli, Rosaria Saletti, Salvatore Foti, Stefania Masci, Francisco Barro. Comparative proteomic analysis of two transgenic low-gliadin wheat lines and non-transgenic wheat control and their implications for gluten intolerances and wheat allergies. *Journal of Proteomics* (under review).

María José Giménez, Ana Real, **María Dolores García-Molina**, Carolina Sousa, Francisco Barro. Characterization of celiac disease related oat proteins: bases for the development of high quality oat varieties suitable for celiac patients. *Nature Scientific Reports* (accepted, 2017).

**María Dolores García-Molina**, Juan García-Olmo, Francisco Barro (2016). Effective Identification of Low-Gliadin Wheat Lines by Near Infrared Spectroscopy (NIRS): Implications for the Development and Analysis of Foodstuffs Suitable for Celiac Patients. *PLoS ONE*, 11 (3).

Francisco Barro, Julio C. M. Iehisa, María J. Giménez, **María Dolores García-Molina**, Carmen V. Ozuna, Isabel Comino, Carolina Sousa, Javier Gil-Humanes (2016). Targeting of prolamins by RNAi in bread wheat: effectiveness of seven silencing-fragment combinations for obtaining lines devoid of coeliac disease (CD) epitopes from highly immunogenic gliadins. *Plant Biotechnology Journal*, 1-11.

Aportaciones a congresos y cursos:

**María Dolores García-Molina**, Juan García-Olmo, Francisco Barro, 2016. Identificación de líneas de trigo con bajo contenido en gliadinas mediante el uso de la Espectroscopía del Infrarrojo Cercano (NIRS). *V Congreso Nacional de la Sociedad Española de Enfermedad Celíaca*. Barcelona, España, 17-19 noviembre.

María José Giménez, **María Dolores García-Molina**, Ana Real, Carolina Sousa, Francisco Barro, 2014. Identification of oat accessions by means of reverse phase high performance liquid chromatography. *IV Congreso Nacional de la Sociedad Española de Enfermedad Celíaca*. Valencia, España, 27-29 noviembre.

**María Dolores García-Molina**, Juan García-Olmo, Francisco Barro, 2013. Determinación de parámetros de calidad de variedades de trigo con muy bajo contenido en epítopos de celiaquía mediante NIR. *X Reunión de la Sociedad Española de Cultivo In Vitro de Tejidos Vegetales*. Zaragoza, España, 22-24 octubre.

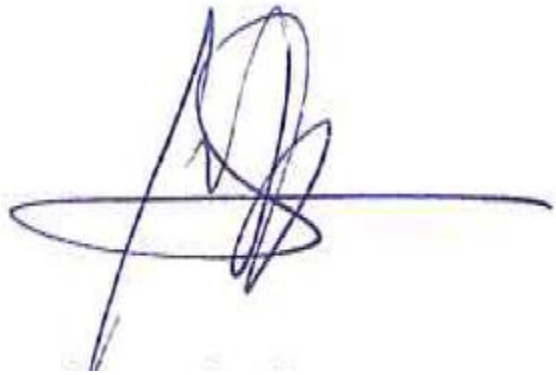
**María Dolores García-Molina**, Juan García-Olmo, Francisco Barro, 2013. Utilidad del NIR para la determinación de parámetros de calidad de trigo para celíacos. II Congreso Científico de Investigadores en Formación en Agroalimentación de la eidA3. Córdoba, España, 9-10 abril.

Curso de Espectroscopía de Infrarrojo Cercano (NIRS), 2013. Aplicaciones en el control de calidad y trazabilidad de productos y procesos (13ª edición). Córdoba, España, 11-14 febrero.

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

Córdoba, 17 de enero de 2017

Firma del/de los director/es



Fdo.: Francisco Barro Losada

*"Si buscas resultados distintos, no hagas siempre lo mismo"*

*Albert Einstein*

*"¿Por qué esta magnífica tecnología científica, que ahorra trabajo y nos hace la vida más fácil, nos aporta tan poca felicidad? La repuesta es esta, simplemente: porque aún no hemos aprendido a usarla con tino"*

*Albert Einstein*

**Agradecimientos**

¿Cómo resumir en unos pocos párrafos todo lo que he vivido durante este período en esta ciudad tan bonita y llena de personas tan maravillosas…? Será difícil, dado que soy propensa a hablar demasiado, pero ahí va mi pequeño grano de arena para todas las personas que han formado parte de esta etapa de mi vida y espero que sigan formando en las sucesivas. En primer lugar me gustaría agradecer a mi director de tesis, Francisco Barro, la oportunidad que me brindó al permitirme formar parte de este equipo, la confianza que depositó en mí desde el principio y, sobre todo, su paciencia a la hora de discutir temas de trabajo conmigo (que prácticamente se lo discuto todo ☺). Además, no puede faltar hacer mención a esa súper tortilla de patatas que nos comemos los viernes para desayunar y que siempre paga él. También me gustaría agradecer a Juan García Olmo por el buen trato que he recibido siempre y por todas las horas de enseñanza acerca del NIR.

Este párrafo está dedicado a todas las personas que están en ese pueblo de locos que casi nadie conoce llamado Albox (mi pueblo) y principalmente a mi familia. Muchas gracias "family monster" por apoyarme siempre, por saber que por muy mal que me vayan las cosas siempre puedo contar con vosotros y por hacer que siempre vea una pequeña luz al final del túnel. También quiero agradecer a mis dos grandes amigas de la infancia, Sandra y Pili, su apoyo incondicional y esas laaaaargas charlas con el propósito de resolver todos nuestros "problemas" y que siempre acaban con un abrazo y una sonrisa. Os quiero mucho.

También me gustaría agradecer a mis Cármenes: a mi paraguaya preferida, Carmen Ozuna, que sin ella mi transcurso aquí habría sido muy diferente. Madre mía, ¿cuántas veces hemos hablado de lo que pondríamos en los agradecimientos de la tesis? "bla bla bla", y ahora…¡¡¡ya ha llegado la hora!!! ¡Qué ilusión! Y lo mejor de todo, compartir tanto buenos como malos momentos contigo y contar con tu "hombro" siempre. Te RE-quiero; y a mi "arcoseña" de los pies a la cabessssa Carmen Calderón, mi clon en número de palabras que necesitamos decir a lo largo del día (que son muchas) y con la que he tenido el gusto de vivir durante 3 años, compartiendo todos nuestros momentos y disfrutando mucho de ese amor fraternal que nos tenemos. Te quiero Car.

A tod@s l@s compis del laboratorio: Ana, muchas gracias por emocionarte cuando todo me salía bien y animarme cuando las cosas no iban tan bien; María José, tu cariño y

disposición para ayudarme en cualquier momento del día no tiene precio. A ambas os agradezco lo querida que me he sentido desde que os conozco. Tadeo, nuestras charlas intentando arreglar el mundo y algunas que otras lecciones de moral no han tenido desperdicio; Fidelaco, nuestra media hora para "tetechar" era el mejor momento del día, ¿verdad? Además, tus clases magistrales sobre *Conyzas* y la búsqueda de sus especies por los campos del IAS, no los cambiaría por nada. Susana gracias por estar siempre dispuesta a ayudar y alegrarte de mis logros.

A Omar, la gran persona que tengo a mi lado día a día y que me hace ver que lo negro puede ser gris clarito. Gracias por no salir corriendo en este pico de ansiedad pre-defensa de tesis, por esa paciencia enorme y esos súper platos culinarios que preparas (que con el estómago lleno todo se ve de otro color☺). Por tu apoyo incondicional y, simplemente, por ser tú.

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A toda la gente de Italia que me ha acogido e integrado en sus vidas. En especial Anna y Giancarlino, mi familia italiana. Tuve muchísima suerte de conoceros y quiero agradeceros la ayuda inmensa que me habéis aportado y el poder contar con vosotros en cualquier momento y para cualquier situación. Vi voglio tantissimo bene e mi mancate molto. Indudablemente en mis agradecimientos no pueden faltar Stefania Masci y Renato D'Ovidio, quienes me trataron como a una hija y me regalaban chocolatitos para animarme cuando los experimentos no salían bien. A Davide por su simpatía y su afán de proponer actividades, además, por estar siempre dispuesto a ayudarme y plantear soluciones mágicas a cualquier tipo de imprevisto; Francesco, Anna, Silvio, Giulia, Alessandra, Peter, Linda, Samuela…. A todos, muchas gracias por hacer que mis estancias en Italia hayan sido espectaculares.

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sobreexpresión de sentimientos en pequeñas fracciones de tiempo☺. ¿Qué te digo que ya no sepas? Simplemente que me alegro mucho de haberte conocido; A Elena, la primera turolense que conozco y encantadísima de que forme parte de mi vida; A Danonino, el gruñoncito más entrañable, que aunque al principio no quería ser mi amigo porque le sobraban amigos, después dejó un hueco para mí y salió una muy bonita amistad; A Álvaro (mi hermanillo), una de las personas más nobles que conozco y un magnífico amigo; A Manuel (el Doc) por su salero y por aceptar tan bien cuando lo excluimos por su posición superior; A Manolillo que no me hace caso y se baña en Australia con tiburones; A Valle por esa sonrisa que siempre lleva cuando nos encontramos por los pasillos; Mercedes, Héctor, Almudena, Fran, Óscar, Carlos……..y a todos aquellos que de un modo u otro habéis hecho que mi estancia aquí haya sido maravillosa.

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**Summary**

Wheat is the world's most produced cereal due to its adaptability to different environments, its high yield, and the biomechanical properties of its flour. Although proteins are present in a lower proportion (9-15%) when compared to starch (60-75%), they are very important for the functionality of the plant. Those proteins can be classified into gluten proteins (80-85%), responsible for the bread-making quality, and non- gluten proteins (10-15%) with a structural function. Then again, gluten proteins also named prolamins due to their high content in proline and glutamine, are made up of gliadins (α-, ω-, γ-), responsible of the extensibility and viscosity of wheat dough, and glutenins (HMW-GS and LMW-GS) which contribute to wheat dough elasticity. However, those prolamins are related to two different enteropathies that affect more that 7% of the global population: celiac disease (CD) and non-celiac wheat sensitivity (NCWS). CD is the most studied pathology and is caused by gluten ingestion, not only wheat but also oat, barley and rye. People with both pathology types need a strict gluten-free diet for their whole life. Nevertheless, this diet is hard to follow because gluten is a widespread additive in the food industry. In fact, diet transgressions are very common and could affect around 32-35% of celiac patients. Furthermore, a gluten-free diet may affect intestinal mucosa leading to a reduction of the beneficial bacteria.

Also, the CD has a genetic component, having a higher risk in the genes that codify the Human Leukocyte Antigen (HLA) DQ2 or DQ8. Wheat gliadin genes are located on the short arm of chromosomes 1 and 6 and are organized in blocks, thus they are inherited as a single locus. Besides, single gene sequences inside the same gliadin family are very similar and can have multiple and different epitopes recognized by T cells. This high level of complexity and the fact that gliadin genes are inherited in blocks makes hard obtaining wheat varieties with a low content of cell T stimulating sequences using conventional breeding techniques.

The development of biotechnology techniques like genetic transformation and interference RNA (RNAi), allowed silencing genes of α-gliadins, γ-gliadins and all gliadins present in wheat grains. The development of varieties with a reduced toxic profile could help to enhance the diet of celiac people and reduce the incidence of CD because it has been observed that the CD is related to the level and duration of gluten

exposure. The study of those lines with low-gliadin content have been the goal of the present thesis because the comprehensive analysis of the proteome will give us information about the possible use of the above mentioned low-gliadin wheat lines in the development of suitable food for celiac and wheat sensitive people.

Through the study of the routes and synthesis mechanisms, folding and deposition of protein bodies, Gil-Humanes et al., observed that wheat lines with all gliadins silenced had a different protein composition when compared to the wild-type, since they had a greater globulin content; however those changes did not affected neither the total protein content nor the stability of the different protein fractions. Those globulins, together with the albumins, form the protein fraction not related to wheat's gluten, having a structural and a metabolic function. According to this information, a comparative proteomic analysis was performed between two transgenic low-gliadin wheat lines obtained using RNAi and non-transformed wheat used as control. The scope of the analysis was to evaluate the silencing target of the genes and their possible effects on the accumulation of other wheat proteins. Because of the great complexity of the wheat proteins, the analysis of each protein fraction was performed separately: gliadins, metabolic proteins and soluble protein in chloroform/methanol (CM-like), using two-dimensional electrophoresis followed by liquid chromatography- mass spectrometry (RP-HPLC/nESI-MS/MS).

The protein accumulation during grain filling is strongly influenced by nitrogen (N) fertilization. The correct fertilization to obtain those lines with reduced toxic profiles is not well known. Adequate fertilization can produce modification on the protein profile, according to that, using different concentrations of N and sulfur (S), both related in the activation/repression of the regulatory elements involved in the expression of these proteins of the wheat grains, we will be able to choose what nutrient concentration is the adequate to obtain a reduction in gliadin content and a good yield. Basing on that, gluten proteins were characterized in lines with a low-gliadin content submitted to different N concentrations and distinct fertilization strategies. Two experiments were performed; in the Experiment 1, three N levels (120, 360 and 1080 mg N) were added at sowing together with two S levels (8 and 30 mg); in the Experiment 2, two levels of N were used (120 and 1080 mg N) which were added according to the time in which the demand for this nutrient was higher, using split applications. Protein quantification was

performed using the RP-HPLC, and the gluten content (ppm) was obtained using the monoclonal antibody R5 (ELISA competitive R5).

A system able to distinguish between wheat lines non-genetically modified and wheat lines with low-gliadin content obtained with RNAi, is necessary to fast and effectively identification of those samples that could be used in the development of suitable products for celiac people. The Near Infrared Spectrometry (NIRS) is a technique that uses near-infrared region of the electromagnetic spectrum (800-2500 nm). The absorption bands are produced when NIR radiation vibrates as the same specific frequency (wavelength) as the molecular bonds of the analyzed sample, being the involved bonds: C-H, N-H and O-H. As a result, each sample has a unique profile, known as "footprint". The discriminant analysis was performed in whole and ground grains. The transgenic samples included 409 samples as whole grain and 414 as flour, while the set of non-genetically modified samples was 126 and 156 for grain and flour, respectively. Besides, the calibration equation was validated twice using a set of samples non-used during calibration. The samples used for the first and second validation were from 2013 and 2014 respectively. The advantages of this spectroscopy rely on its non-destructive technique, requiring little sample amounts, being able to produce fast and reliable results, which in turns produces a reduction in time and costs.

i. The comparative analysis of the proteome of the low-gliadin wheat lines (D783 and D793) and its control BW208 with two-dimensional electrophoresis and mass spectrometry, showed that HMW-GS, metabolic proteins and soluble proteins in chloroform/methanol were more present in the transgenic lines analyzed, especially in D793 which silencing promoter was more effective. Based on these data and considering that metabolic proteins and those soluble in chloroform/methanol, such as α-amylase, β-amylase and serpins, are related to food and respiratory wheat allergens, it may be concluded that these lines with low-gliadin content could be used for the development of food suitable for celiac, but not for those people with wheat allergy.

ii. The results relative to the best fertilization for low-gliadins wheat lines were different depending on the fertilization strategy applied. It was demonstrated that the efficiency of the promoter in the silencing of gliadins is directly related to protein accumulation. Thus, the lines D793 and E82 showed low gliadin content and an

increase in the glutenin content with increasing N. The ELISA Competitive analysis with the monoclonal antibody R5 showed a significant decrease in gluten content (ppm) using split N fertilizations (Experiment 2) when compared with the results obtained in Experiment 1 using 120 mg N, which may suggest that the best fertilization strategy to diminish the toxicity will be the one used in Experiment 2. Furthermore, line E82 ensures that variations in the N fertilization would not produce an increase in gluten content.

iii. The traceability in the commercialization of gluten free products is essential to ensure that those products do not suffer cross contamination and can be consumed by celiac patients and wheat sensitive people. We have demonstrated that the discrimination between lines with low-gliadin content and non-transgenic wheat is possible using the NIR technology, developing a robust model for the classification of those lines. Besides, the analysis was performed in whole and ground grains, having better results when the samples were in the form of flour, because the 99% of the flour samples and the 96% of the grain samples used during the validation, were correctly classified.

**Resumen**

El trigo es el cereal más cultivado en el mundo debido a su adaptabilidad a diferentes ambientes y a su alto rendimiento, así como por las propiedades biomecánicas que presenta su masa. Aunque las proteínas del trigo están en menor proporción (9-15%) comparado con el almidón (60-75%), son muy importantes para su funcionalidad. Estas proteínas pueden clasificarse en proteínas del gluten (80-85%), responsables de la calidad harino-panadera; y proteínas no pertenecientes al gluten (15-20%), con función principalmente estructural. A su vez, las proteínas del gluten, también llamadas prolaminas por su alto contenido en prolina y glutamina, están formadas por gliadinas (α-, ω-, γ-), responsables de la extensibilidad y viscosidad de la masa del trigo, y gluteninas (HMW-GS y LMW-GS) que contribuyen a la elasticidad. Sin embargo, estas prolaminas están asociadas con dos importantes enteropatías que afectan a más del 7% de la población: la enfermedad celíaca (EC) y la sensibilidad al trigo no celiaca. La EC es la patología más estudiada y es causada por la ingestión del gluten, no sólo del trigo, sino también de la avena, cebada y centeno. Las personas que presentan ambos tipos de patologías requieren una dieta estricta libre de gluten durante toda la vida. No obstante, esta dieta es difícil de llevar a cabo puesto que el gluten es un aditivo ampliamente usado en la industria alimentaria. Además, las transgresiones en la dieta son muy comunes y podrían afectar al 32-55% de los pacientes celiacos. Por otro lado, la dieta libre de gluten puede perjudicar la salud de la mucosa conduciendo a una reducción de la población de bacterias beneficiosas.

También, la EC tiene un componente genético, presentando un mayor riesgo los genes que codifican el antígeno leucocitario humano (HLA) DQ2 o DQ8. Los genes de las gliadinas de trigo se encuentran localizados en el brazo corto de los cromosomas 1 y 6, estando organizados en bloques, por lo que se heredan como un único locus. Asimismo, las secuencias de los genes individuales, dentro de la misma familia de gliadinas son muy similares, y pueden contener múltiples y diferentes epítopos reconocidos por las células T. Este alto nivel de complejidad, y el hecho de que los genes de las gliadinas se heredan en bloques, hace que sea muy difícil obtener variedades de trigo, con reducido contenido de secuencias estimuladoras de células T utilizando técnicas de mejora genética convencional.

El desarrollo de técnicas biotecnológicas como la transformación genética y el ARN de interferencia (ARNi) han permitido silenciar genes de α-gliadinas, γ-gliadinas y todas las gliadinas del grano de trigo. El desarrollo de estas variedades, con un perfil tóxico reducido, puede contribuir a mejorar la dieta de personas celíacas y reducir la incidencia de la EC, ya que se ha observado que la iniciación de la EC está asociada con el nivel y duración de la exposición al gluten. Estas líneas con bajo contenido en gliadinas han sido objeto de estudio en esta tesis, ya que el análisis exhaustivo del proteoma nos dará información sobre la posible utilización de estas líneas de trigo en el desarrollo de alimentos aptos para el colectivo celiaco y con sensibilidad al trigo.

Mediante el estudio de las rutas y mecanismos de síntesis, plegamiento y deposición de las prolaminas en los cuerpos proteicos, Gil-Humanes et al., 2011 observaron que las líneas de trigo que presentaban todas las gliadinas silenciadas tenían una composición proteica diferente a la del trigo no modificado genéticamente, ya que contenían una mayor cantidad de globulinas, pero estos cambios no afectaban al contenido total de proteínas ni a la estabilidad de las diferentes fracciones. Estas globulinas, junto con las albúminas, componen la fracción proteica no perteneciente al gluten de trigo, presentando función estructural y metabólica. En base a esta información, se realizó un análisis proteómico comparativo entre dos líneas de trigo con bajo contenido en gliadinas obtenidas mediante RNAi y un trigo no transformado usado como referencia. El objetivo de este análisis era evaluar el target de silenciamiento de los genes y sus posibles efectos en la acumulación de otras proteínas del trigo. Debido a la gran complejidad de las proteínas del trigo, se llevó a cabo el análisis de cada fracción proteica por separado: gliadinas, gluteninas, proteínas metabólicas y proteínas solubles en cloroformo/metanol (CM-like), utilizando electroforesis bidimensional seguida de cromatografía líquida-espectrometría de masas (RP-HPLC/nESI-MS/MS).

La acumulación proteica durante el llenado del grano está fuertemente influenciada por la fertilización con nitrógeno (N). El abonado adecuado para obtener estas líneas con perfiles tóxicos reducidos no es del todo conocido. La fertilización adecuada puede producir modificaciones en el perfil de proteínas, de este modo, utilizando diferentes concentraciones de N y azufre (S), macronutrientes implicados en la activación/represión de los elementos reguladores que controlan la expresión de estas proteínas en el grano de trigo, podremos seleccionar qué concentración de nutrientes es la adecuada para obtener una reducción del contenido en gliadinas unido a una buena

producción. En base a esto, hemos caracterizado las proteínas del gluten en líneas que presentan bajo contenido en gliadinas sometidas a diferentes concentraciones de N y diferentes técnicas de fertilización. Se llevaron a cabo dos experimentos; en el Experimento 1 se usaron tres niveles de N (120, 360 y 1080 mg N) añadidos en el momento de la siembra, combinados con dos de S (8 y 30 mg S); el Experimento 2 incluyó dos niveles de N (120 y 1080 mg N) que fueron añadidos de acuerdo a los momentos de mayor demanda por parte de la planta usando aplicaciones fraccionadas. La cuantificación proteica se realizó usando RP-HPLC y el contenido en gluten (ppm) se determinó mediante el anticuerpo monoclonal R5 (ELISA competitivo R5).

Un sistema capaz de distinguir entre líneas de trigo no modificado genéticamente y líneas con bajo contenido en gliadinas, obtenidas mediante RNAi, es necesario para una rápida y efectiva identificación de aquellas muestras que podrán ser usadas en el desarrollo de productos adecuados para las personas celíacas. La Espectroscopía del Infrarrojo Cercano (NIRS) es una técnica que usa la región del infrarrojo cercano del espectro electromagnético (800-2500 nm). Las bandas de absorción se producen cuando la radiación NIR vibra a la misma frecuencia específica (longitudes de onda) que los enlaces moleculares de la muestra analizada, siendo los enlaces involucrados C-H, N-H y O-H. Esto da lugar a que cada muestra tenga un perfil único, que se conoce como "huella". El análisis discriminante se realizó tanto en grano entero como en grano molido. El conjunto de muestras transgénicas incluyó 409 muestras en forma de grano entero y 414 de harina, mientras que el conjunto de muestras no modificadas genéticamente ascendió a 126 y 156 muestras para grano y harina, respectivamente. Además, la ecuación de calibración fue validada dos veces usando un conjunto de muestras que no se emplearon para la calibración. Las muestras usadas para la primera y segunda validación pertenecieron al año 2013 y 2014, respectivamente. Las ventajas de esta espectroscopía es que es una técnica no destructiva que requiere una pequeña cantidad de muestra, capaz de generar resultados de forma rápida y fiable, lo que conlleva a una reducción de tiempo y coste.

i. El análisis comparativo del proteoma de las líneas con bajo contenido en gliadinas (D783 y D793) y su control BW208 mediante electroforesis bidimensional y espectrometría de masas, demostró que las subunidades de gluteninas HMW-GS, proteínas metabólicas y proteínas solubles en cloroformo/metanol se acumularon más en las dos líneas transgénicas

analizadas, especialmente en la D793 cuyo promotor de silenciamiento fue más efectivo. Basándonos en estos datos y considerando que las proteínas metabólicas y aquellas solubles en cloroformo/metanol, tales como inhibidores de α-amilasa, β-amilasa y serpinas, están relacionadas con alérgenos alimentarios o respiratorios del trigo, podemos concluir que estas líneas con bajo contenido en gliadinas podrían ser usadas para el desarrollo de alimentos aptos para celíacos, pero no para el colectivo alérgico al trigo.

ii. Los resultados relativos a la mejor fertilización para líneas de trigo con bajo contenido en epítopos tóxicos fueron diferentes en base a la estrategia de fertilización seguida. Se demostró que la eficiencia del promotor en el silenciamiento está directamente relacionada con la acumulación de las proteínas. Así, las líneas D793 y E82 presentaron un bajo contenido en gliadinas y un incremento en el contenido de gluteninas cuando se aumentó el aporte de N. El análisis ELISA Competitivo con el anticuerpo monoclonal R5 mostró un descenso significativo en el contenido de gluten (ppm) usando aplicaciones fraccionadas de N (Experimento 2) al compararlo con los resultados obtenidos en el Experimento 1 usando 120 mg N, lo que indicaría que la mejor fertilización para disminuir la toxicidad sería aquella referente al Experimento 2. Además, la línea E82 asegura que las variaciones en la fertilización con N no darán lugar a un incremento en la ppm de gluten.

iii. La trazabilidad en la comercialización de productos libres de gluten es esencial para asegurar que dichos productos no sufren contaminación cruzada y podrían ser consumidos por pacientes celíacos y personas con sensibilidad al trigo. Nosotros hemos demostrado que la discriminación entre líneas con bajo contenido en gliadinas y trigos no transgénicos es posible usando la tecnología NIR, desarrollando un modelo robusto para la clasificación de estas líneas. Además, el análisis se llevó a cabo tanto en grano entero como molido, obteniendo mejores resultados cuando la muestra analizada estaba en forma de harina, ya que el 99% de las muestras de harina y el 96% de las muestras en grano, usadas durante la validación, fueron clasificadas correctamente.

# *Chapter 1*

## General Introduction

# General Introduction

## 1.1 Wheat

### *1.1.1 Origin of wheat*

Wheat is one the oldest and most cultivated crops in the world, having a global annual production of about 729 million tons being Asia the largest producer, followed by Europe, America, Africa and Oceania (Figure 1.1). Wheat is among the three most produced cereal in the world, behind corn (1,038 million tons) and rice (741 million tons) (data from 2014; http://faostat3.fao.org). Wheat cultivation dates back to 10,000 years old, as part of the "Neolithic Revolution" and it is considered one of the main reasons for which humans transformed from hunter-gatherer nomad to settled agriculturalist (Shewry, 2009), due to easy seed storage for a long period of time, promoting the development of cultural achievement since it was not necessary to seek food every day.

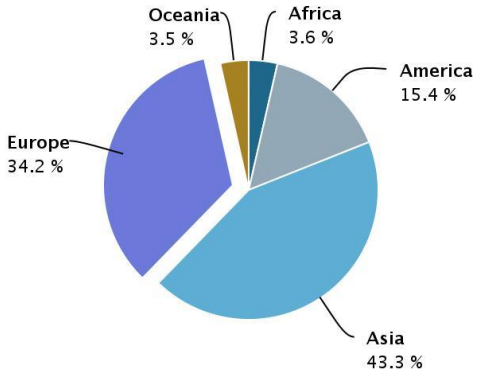


**Figure 1.1.** Worldwide distribution of wheat production.

Wheat denotes the whole grain in either cultivated and wilds belonging to *Triticum* genus. They are annual plants, the Poaceae family, who have undergone a long evolution, being the current wheats the result of natural hybridization between different species. The basic chromosome number is 7. The ancestor of durum wheat, wild tetraploid (genome AABB, 2n=4x=28) (emmer) *Triticum turgidum* subsp. *dicoccoides*, was originated by crossing the wild ancestor of diploid wheat, *Triticum urartu* (genome AA, 2n=2x=14), with an unknown species, probably coming from S genome existent in *Aegilops* (with *Ae*. *speltoides* as closest species), that provided the B genome (BB,

2n=2x=14) (Feldman, 2001). From emmer, current tetraploid wheats such as *Triticum turgidum* ssp. *durum* were developed. The hexaploid bread wheat probably occurred in a field of cultivated emmer (*Triticum turgidum* ssp. *dicoccum*), when this was crossed with *Aegilops tauschii*, which provided the D genome, and giving rise to genome AABBDD (2n=6x=42). The D genome was responsible, not only for the unique bread-making features of hexaploid wheat, but also of the worldwide expansion of bread wheat. Owing to the superior qualities obtained from this hybridization, this wheat was selected by farmers and it has evolved to the present day.

### *1.1.2 Wheat composition*

Wheat is widely consumed in the world, providing, in some countries, the major nutritional source of the diet. It is mainly composed by starch, about 60-75% of the total dry weight, which causes that wheat had been simply considered a source of calories. However, despite its lower protein content (9-15%), the nutritional importance due to these proteins should not be underestimated since a high percentage of protein intended for human and livestock nutrition comes from wheat. Furthermore, thanks to gluten proteins, wheat has unique biomechanical properties ensuring an essential role in the dough functionality.

Wheat grains include two major groups of proteins: gluten proteins (80%) responsible for bread making quality; and non-gluten proteins (20%), comprising metabolic and chloroform/methanol-soluble (CM-like) protein fractions, with structural and metabolic functions having a minor role in bread making (Figure 1.2).

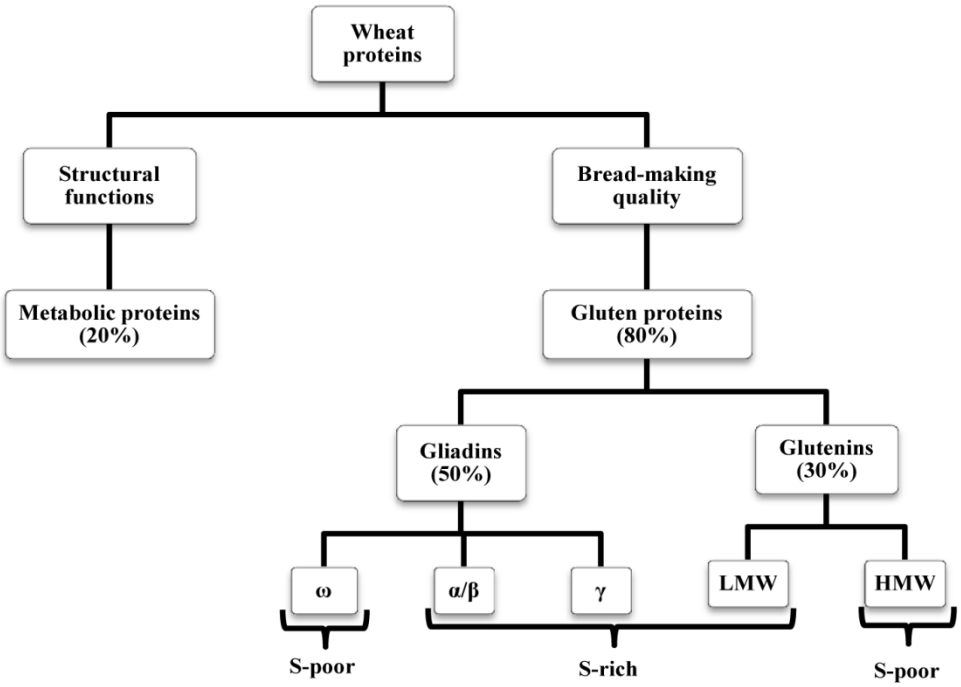


**Figure 1.2.** Classification of wheat proteins related to sulphur content.

Gluten proteins, also called prolamins due to their high content of proline and glutamine (Shewry et al., 1995;Shewry and Halford, 2002), encompass gliadins and glutenins. Gliadins are monomeric and they are classified, on the basis of their electrophoretic mobility in acid-polyacrylamide gels (A-PAGE), in three fractions: α/β-, ω- and γ-gliadin. Within each fraction, there are small differences; ω-gliadins present the highest contents of glutamine, proline and phenylalanine in their composition; α/β- and γ-gliadins differ in the proportions of aspartic acid, proline, methionine, tyrosine, phenylalanine and tryptophan. Moreover, although the distribution of total gliadins will strongly depend on genotype and environmental conditions, generally α/β- and γ-gliadins are major components, while the ω-gliadins are in minority (Wieser and Kieffer, 2001). On the other hand, glutenins comprise two major groups, classified in function to their electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE); the high molecular weight subunits of glutenins (HMW-GS) and the low molecular weight subunits of glutenins (LMW-GS). LMW-GS are the most abundant proteins in the glutenin fraction and they are classified into three groups based on electrophoretic mobility in SDS-PAGE (Jackson et al., 1983): groups B and C (Payne and Corfield, 1979) and additional group denominated D. Masci et al., 1999 (Masci et al., 1999) demonstrated that D group is mainly composed by ω-gliadins components

which have acquired a cysteine residue. Moreover, a later study revealed that the group C is formed by LMW-GS with terminal sequences of α/β- and γ-gliadins (Masci et al., 2002). Finally, group B comprises the overriding majority of typical LMW-GS, although there is a little proportion of typical sequences of gliadins (Masci et al., 2002). The HMW-GS belong to the minor components within the gluten protein family. The nomenclature of this group is based on the coding genome (A, B or D) and the type *x* or *y* according to electrophoretic mobility, being *x-type* the slowest and *y-type* the fastest (Shewry et al., 1992).

Gliadins confer extensibility and viscosity to the doughs, but it is very complicated to explain the role of each gliadin fraction in the properties of the mass, since genes encoding gliadins are inherited in blocks. Moreover, some genes encoding LMW-GS and gliadins are closely linked and can be attributed to gliadins properties of glutenins. On the other hand, glutenins are responsible for elasticity and the formation of long polymers linked by disulphide bonds, contributing especially HMW-GS to these properties (Shewry et al., 2003); nevertheless, LMW-GS have been associated with the strength and extensibility of the dough (Metakovsky et al., 1990).

Disulphide bonds play an important role in determining the structure and properties of gluten protein, therefore, the amino acid cysteine is very important for the functionality of gluten (Grosch and Wieser, 1999). Most ω-gliadins cannot establish disulphide bonds due to the lack of cysteine in their composition; however, α/β- and γ-gliadins contain six and eight cysteine residues, respectively, so they form intra-chain crosslinks (Grosch and Wieser, 1999). LMW-GS, α/β- and γ-gliadins present a similar amino acid composition. In addition to the intra-chain disulfide crosslinks, LMW-GS have two cysteine residues that are not capable of forming intramolecular bonds, but intermolecular bonds with other gluten proteins. Consequently, another way of classifying gluten proteins is based on their sulphur content (Shewry et al., 1986). Hence, S-rich fractions comprised α/β- and γ-gliadins, and LMW-GS; S-poor fractions correspond to ω-gliadins; and HMW-GS prolamins with an intermediate proportion between those of the S-poor and S-rich fractions (Figure 1.2).

The biomechanical properties of wheat, usually called viscoelasticity, are due to the formation of intra and intermolecular bonds by stabilizing gluten, and forming very large polymers, which allows the encapsulation of carbon dioxide during fermentation

and the expansion of the dough, providing the typical features of volume and texture of bread and other baked products. Moreover, the amount and composition of the gluten proteins also differ among genotypes and with the environment, leading to differences in dough properties, which are used to make a wide range of products including cakes, pasta, bread, noodles and biscuits.

The exceptional characteristics of gluten proteins have provided that gluten is widely used by industry, appearing in a wide range of products which originally do not contain gluten such as meat, fish and many other foodstuffs. Gluten proteins, and also non-gluten proteins, are related to important human enteropathies and allergies such as, celiac disease (CD), non-celiac wheat sensitivity (NCWS), wheat dependent exercise induced anaphylaxis (WDEIA) and baker's asthma.

### 1.2 Celiac disease

This is the best known of all gluten related disorders. It can be defined as an autoimmune inflammation of the small intestine that occurs in genetically predisposed individuals, after ingestion of certain peptides from wheat (gliadins and glutenins), barley (hordeins), rye (secalins), oats (avenins) and hybrids of these cereals such as triticale. Therefore, it is difficult to determine the origin of CD since it has a strong environmental component and a strong genetic component, presenting a variant of human leukocyte antigen (HLA)-DQ2 and HLA-DQ8 (Sollid, 2002). These cereals contain toxic epitopes where the deamination of glutamine by the action of tissue transglutaminase 2 (tTG2) is essential to promote the link between both HLA-DQ2 and/or HLA-DQ8 and recognition of cell-T cluster of differentiation 4 (T $CD4^{+}$) which cause the changes in duodenal and jejunal mucosa, leading to a cascade of inflammatory reactions by releasing cytokines that interfere with absorption of nutrients. The HLA-DQ2 antigen is found in about 95% of celiac patients, while only about 5% of patients present the HLA-DQ8 and 10% of celiac patients have both antigens (Karell et al., 2003). Strict adherence to a gluten free diet leads to rapid and complete recovery of the architecture and function of the intestinal mucosa. However, since this enteropathy is also produced by the ingestion and exposure to gluten from barley, and rye (Trier, 1998), increasing the transgressions risk in gluten free diets, by between 32 and 55% of CD patients (Silvester and Rashid, 2007).

### 1.3 NCWS

There is no unambiguous definition that describes the NCWS, as it is a very heterogeneous disease, whose symptoms are apparently caused by various mechanisms (Di Sabatino et al., 2013). It can be said that is a reaction to gluten proteins from wheat, in which allergic and autoimmune mechanisms have been excluded. Actually, it is hypothesized that other proteins such as metabolic proteins called α-amylase/trypsin inhibitors (ATI) (Junker et al., 2012), and FODMAPS (Fermentable Oligo-saccharides, Disaccharides, Monosaccharides and Polyols) play an important role in the development of this pathology. Individuals present a normal small-bowel mucosa and their intestinal or no intestinal symptoms can improve or disappear by removing wheat from the diet.

### 1.4 WDEIA

It is a form of allergic reaction caused by ingestion of wheat with subsequent physical exercise. Wheat or neither exercise alone nor provoke the reaction; the combination of both is required. The major allergens associated with this disease are the ω5-gliadins (Palosuo et al., 2001b;Morita et al., 2003). Palosuo et al., 2003 (Palosuo et al., 2003) suggested that the development of large allergen complexes responsible for triggering anaphylactic reactions could be related to activation of transglutaminase in the intestinal mucosa during the physical exercise in patients with WDEIA. Moreover, the presence of crossed allergenic reactions in rye and barley has been described by Palosuo et al., 2001 (Palosuo et al., 2001a).

### 1.5 Bakers' asthma

This respiratory allergy is elicited by a wide range of wheat proteins which react with immunoglobulin E (IgE) from patients with bakers' asthma. In this group of proteins we could include gliadins, glutenins, serine proteinase inhibitors (serpins), thioredoxin, agglutinin and several enzymes (Tatham and Shewry, 2008). Among these enzymes, types of α-amylase inhibitors have been described as the major group of proteins responsible to cause the bakers' asthma, moreover, these wheat enzymes belong to group of proteins soluble in chloroform:methanol (CM-like proteins) (Salcedo et al., 2007).

### 1.6 Wheat lines with low-gliadin content

The development of wheat varieties without toxic epitopes has been studied for a long time. Spaenij-Dekking et al., 2005 (Spaenij–Dekking et al., 2005) and van Herpen et al., 2006 (van Herpen et al., 2006) showed the existence of wheats with low or no content of celiac toxic epitopes, suggesting that selection of less toxic lines through classical breeding could be possible. However, gliadin genes of wheat are located on the short arm of chromosomes 1 and 6, being organized into blocks, which are inherited as a single locus. Furthermore, the sequences of individual genes within the same family of gliadin are very similar, and can contain multiple and different epitopes recognized by T cells (van Herpen et al., 2006). This high level of complexity and heritability in blocks makes it virtually impossible the development of wheat varieties suitable for people with celiac disease using classical breeding.

The use of biotechnology techniques such as RNA interference (RNAi) has allowed to down-regulate the expression of particular gliadin fractions (Gil-Humanes et al., 2008;Altenbach and Allen, 2011;Becker et al., 2012) and total gliadins (Gil-Humanes et al., 2010) in the grain of wheat. Hence, reducing toxic epitopes may result in a decrease of the incidence of the pathologies related to gluten protein given that have been associated the development of celiac disease with the amount and exposure to gluten (Ventura et al., 1999). The ability of wheat lines with all gliadins silenced to stimulate T-cell clones from celiac patients was evaluate and it was obtained a reduction in proliferative response for some transgenic lines (Gil-Humanes et al., 2010). In spite of the gluten protein composition has been changed, the total protein content was equivalent to that of the wild type (Gil-Humanes et al., 2010;Gil-Humanes et al., 2011;Pistón et al., 2011;Barro et al., 2015) suggesting a compensation mechanism with other proteins like albumins and globulins (Gil-Humanes et al., 2011). Moreover, rheological properties determinations were carried out and these transgenic lines presented higher stability and tolerance to over-mixing (Gil-Humanes et al., 2014). Several studies have tried to establish the amount of gluten that celiac people can tolerate. Catassi et al., 2007 (Catassi et al., 2007) reported that 50 mg gluten per day is the minimum dose required to produce measurable damage to the small-intestinal mucosa in CD patients. Taking into account this requirement, celiac people could consume about 67 grams of bread elaborated with low-gliadin flour per day, and this amount could be eve increase by mixing with other gluten free cereals like rice or

maize. Therefore, these low-gliadin wheat lines could be used by celiac and wheat sensitive patients, or by people that prefers a low gluten diet, even in absence of any symptom related to adverse reactions to wheat. Even more, low-gliadin lines can help to improve the nutritional components of the GFD, because a full GFD might not provide suitable quantity of proteins, vitamins, minerals and fiber, it might be detrimental to gut bacteria microbiota (De Palma et al., 2009). Furthermore, due to the unique properties of wheat gluten to form a viscoelastic protein network, gluten free products are made with high amounts of fat and sugars in order to achieve a similar texture to that obtained with wheat.

### 1.7 Proteomic studies to identify allergens in wheat

Proteomic is an approach that allows us to know gene expression of biological systems by analyzing polypeptides. Depending on development stage of crop and environmental conditions, protein expression can vary greatly (Wrigley et al., 2003). Currently, the most used strategies to isolate, characterize and identify proteins are the combination of two-dimensional electrophoresis with mass spectrometry (2-DE/MS) or with immunoblotting to identify IgE against a range of wheat proteins (Laino et al., 2010;Larré et al., 2011;Hurkman et al., 2013;Lupi et al., 2013;Lupi et al., 2014). 2-DE combined to MS has been widely utilized to compare the proteome of transgenic lines with their non-transgenic controls (Brandão et al., 2010;Lupi et al., 2013;Lupi et al., 2014). The 2-DE separates the proteins in "two dimensions". The separation during the first dimension or isoelectric focusing is reached by focusing the solubilized proteins in a gel that contents an immobilized pH gradient (IPG strips). Proteins migrate and remain where their net charge is zero (isoelectric point). In second dimension, the IPG strip is equilibrated in a buffer with sodium dodecyl sulfate (SDS) which denaturizes the proteins providing a constant mass/charge relation to allow the subsequent separation according to their molecular weight. On the other hand, mass spectrometry (MS) is a tool to identify the proteins. Mass spectra provide structural information about complexes molecular species, isotopic relationships between atoms and samples and qualitative and quantitative composition of organic and inorganic analytes. This approach has been successfully applied in different species, wheat included, since the comparative proteome analysis allows the understanding of the changes that occur in the plant in response to environmental factors or genetic modifications. Proteomic studies have been carried out during the immature and mature grain giving rise to thousands of

components (Rathmell et al., 2000). Many proteins identified in mature and immature grain correspond to prolamins (gliadins and glutenins). Nevertheless, the majority of proteins present in mature grain belong to structural and metabolic proteins, such as α-amylase inhibitors, trypsin inhibitors, heat-shock and ribosomal proteins, and isoforms of superoxide dismutase (Skylas et al., 2005). There is a long list of authors who have established a direct relationship between wheat proteins and specific human allergies. Lupi et al., 2013, 2014 (Lupi et al., 2013;Lupi et al., 2014) assessed the transgenesis implication in the wheat allergenicity using transgenic lines and their counterparts. Moreover, Sestili et al., 2013 (Sestili et al., 2013) carried out the comparative analysis of two transgenic lines obtained with different transformation techniques to determine if the protein results differed between procedures. In 2014 Altenbach et al., (Altenbach et al., 2014), developed transgenic lines with reduced level of ω-5 gliadins and they carried out the protein comparison between two wheat transgenic lines and a control. The results showed that ω-5 gliadins are not only the major proteins involved in WDEIA, but also their content increase during the development of grain caused by environmental changes resulting in a decline on flour quality.

### 1.8 Effects of fertilization in wheat grain composition

The filling of the grains and their subsequent germination require energy and nutrients that will be contributed through the mobilization of specific groups of storage components and the digestion of storage organs. In this way, changes in the quantity and composition of these storage components will result in impacts on yield and characteristics in the final use of the grains.

Mineral fertilization has major contribution to the synthesis of wheat grain compounds. Specifically, nitrogen (N) and sulfur (S) necessarily influence the synthesis of storage proteins given its role in the amino acids structure. Hence, S is part of the composition of cysteine, amino acid responsible to form disulphide bonds intra or inter-chain originating the gluten functionality characteristics. On the other hand, N is related to yield, being key factors the source, amount and timing of N applications. Moreover, most of the N in grain is in the form of proteins resulting in high glutamine content (Shewry, 2011). There are discrepancies in the results reported by different authors related to N fertilization (Ayoub et al., 1994;López-Bellido et al., 1998;Garrido-Lestache et al., 2004;2005). This may be because the methodologies used to analyze and

quantify gluten proteins are different because of the difficulty in analyzing these proteins. In addition, variations in protein composition depend not only of N input but also by N application techniques, genotype and environmental conditions. When only N fertilization effects are considered, N available for wheat will determine the correlations between storage and non-storage proteins, both ratios gliadin:glutenin and HMW:LMW. Moreover, N and S may interact altering their availability because an N:S ratios greater than 17:1 would decrease S uptake resulting in low formation of cysteine and methionine amino acids and the appearance of non-nitrogenous proteins as amides (Wrigley et al., 1980). Therefore, deficiency of these minerals can affect both the quantity and composition of storage compounds, and consequently, to the appropriate balance between the viscosity and extensibility conferred by gliadins and the elasticity provided by glutenins, required to obtain a good quality of the final product.

A more comprehensive understanding of how fertilization affects the composition of grain proteins could simplify the challenge of getting the trinomial protein quality, high yield and good N use efficiency.

### 1.9 Near Infrared Spectroscopy (NIRS)

It is a spectroscopy method using near infrared region of the electromagnetic spectrum (800-2500 nm). The absorption bands in this region are overtones and combinations of vibrational bands. These absorption bands are produced when the NIR radiation vibrates at the same specific frequency (wavelengths) than the molecular bonds of the analyzed sample, and therefore, each sample has a unique absorption profile for that sample, which is known as spectral fingerprint. Usually, the chemical bonds involved are: C-H, N-H, and O-H (Figure 1.3).

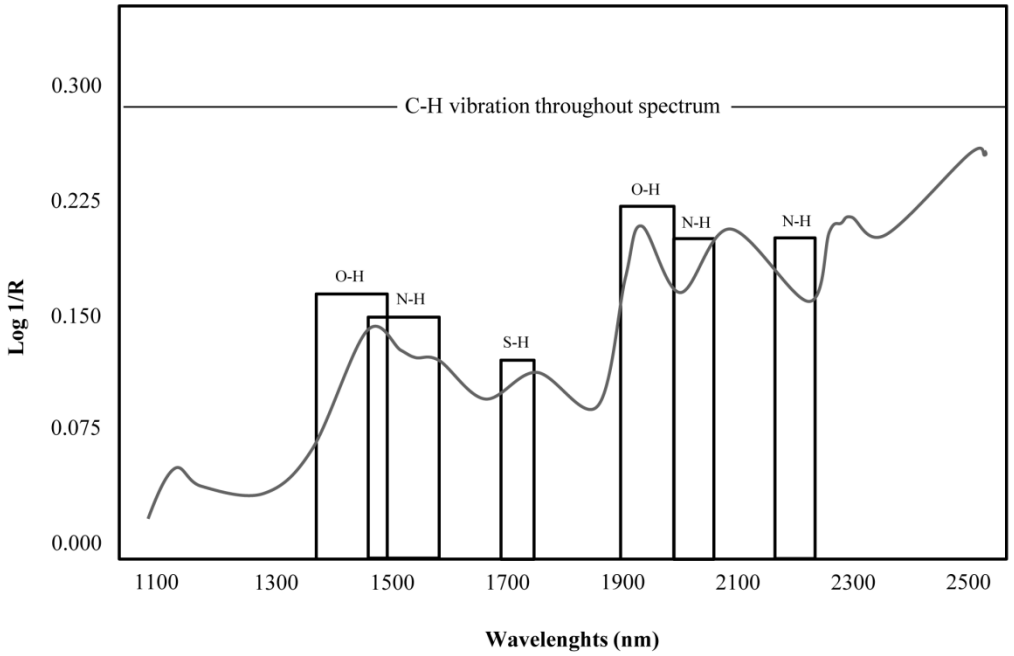


**Figure 1.3.** Diagram of the vibrational bonds with hydrogen (X-H) in agricultural products. Grey line represents the NIR spectrum. Image adapted from Shenk and co-workers, 2001 (John et al., 2001).

Concentrations of components such as water, protein, fats and carbohydrates could be determined using classical absorption spectroscopy. However, the particle size is responsible for changes in spectra derived from the chemical information of most foods. As a consequence, the NIR method depends on a reference method in order to obtain a good calibration of the measured parameter.

In the case of powder samples such as wheat flour, the calibration curve would be obtained by diffuse reflectance using multivariate mathematic (chemometries) approaches. The determination of compounds of powder samples by diffuse reflectance occurs as follows: i) the radiation penetrates the surface; ii) reaches each particle of the sample; iii) this radiation can be reflected, absorbed or transmitted. The result is that reflected radiation ($R$) can be empirically related to concentration ($c$) in an analogous way to Beer's law ($\log 1 / R = kc$, where k is a factor incorporating absorbance and the length of the path) (Osborne, 2006b). Figure 1.4 shows the NIR diffuse reflectance model. The powder sample is placed inside a capsule one cm deep with a quartz window. The capsule containing the sample is introduced into the instrument where it

will be irradiated with NIR radiation. The reflected radiation that will carry the information of the sample will be captured by a set of detectors.

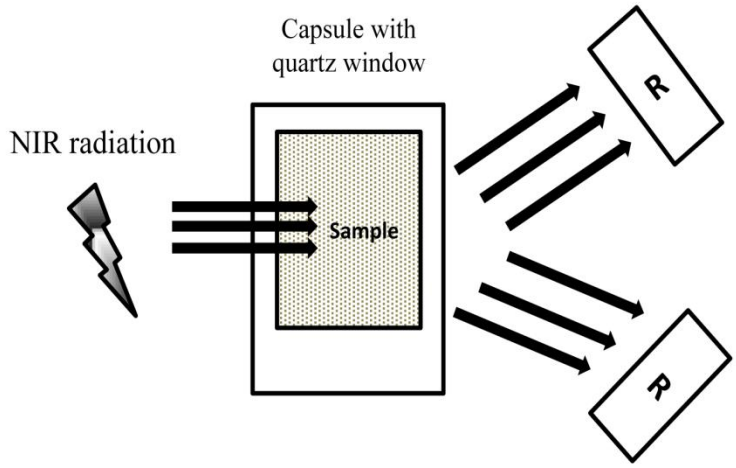


**Figure 1.4.** Obtaining information from a powder sample using diffuse reflectance captured by detectors.

NIRS is routinely used for the composition, functional and sensory analysis of food ingredients, intermediate processes and final products. The main advantage of NIR is that it normally does not require sample preparation, so the analysis is easy and fast and can be carried out online. Moreover, NIR technology can measure several constituents simultaneously.

NIRS has been utilized by researchers to detect the cereal quality factors that are interesting for the breeders (Osborne, 2006a), including hardness, flour yield, starch, water absorption, develop time of the dough, extensibility, bread volume, dough changes during mixing (Alava et al., 2001;Aït Kaddour et al., 2008a;Aït Kaddour et al., 2008b), and durum wheat adulteration (Cocchi et al., 2006). In addition to quantitative analysis, qualitative analysis by NIRS chemometric techniques has been used to distinguish groups of samples with common characteristics and successfully applied to verify the origin of agricultural products. Thus, this method has been used as a valuable tool for quality control in the food industry to predict and discriminate foods components related to human health, such as acrylamide and mycotoxins present in cereal grains (Segtnan et al., 2006;De Girolamo et al., 2009), and especially in grains and flours (Osborne et al., 1993).

## 1.10 Objectives

In this thesis we have characterized the low-gliadin transgenic wheat lines for the following specific objectives:

The characterization of the proteome of two low-gliadin wheat lines obtained by RNAi using two different endosperm specific promoters to evaluate the effects on target and non-target genes and implications on the expression of other kernel proteins.

To determine how fertilization strategies combined with different nitrogen and sulphur inputs affect the protein profile of low-gliadin wheat lines generated by RNAi, especially those proteins responsible for celiac disease.

To develop NIRS discrimination models for the effective identification of low-gliadin wheat samples created by RNAi, from wheat lines containing the full set of gliadins, in both whole grain and flour.

The thesis is presented in chapters, which have the structure used by peer-reviewed publications. Chapters two and three are under review. Chapter four was published in the journal PLoS ONE. Each chapter coincides with the specific objectives defined above.

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# *Chapter 2*

## Comparative proteomic analysis of two transgenic low-gliadin wheat lines and non-transgenic wheat control and their implications for gluten intolerances and wheat allergies

**Comparative proteomic analysis of two transgenic low-gliadin wheat lines and non-transgenic wheat control and their implications for gluten intolerances and wheat allergies**

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**Abstract**

Gluten proteins are major determinants of the bread making quality of wheat but also of important gluten-related disorders, including celiac disease (CD), and allergies. We carried out a proteomic study using the total grain proteins from two low-gliadin wheat lines, obtained by RNAi, and the untransformed wild type as reference. The impact of silencing on both target and on non-target proteins was evaluated. Because of the great protein complexity, we performed separate analyses of four kernel protein fractions: gliadins and glutenin subunits, and metabolic and CM-like proteins, by using a classical 2D electrophoresis gel based approach followed by RP-HPLC/nESI-MS/MS.

As a result of the strong down-regulation of gliadins, the HMW-GS, metabolic and chloroform/methanol soluble proteins were over-accumulated in the transgenic lines, especially in the line D793, which showed the highest silencing of gliadins. Basing on these data, and considering that metabolic proteins and chloroform/methanol soluble proteins (CM-like), such as the α-amylase/trypsin inhibitor family, β-amylase and serpins, were related to wheat allergens, these transgenic lines could be suitable for celiac or other gluten intolerance patients but not for allergic people.

Biological significance

Several enteropathies and allergies are related to wheat proteins. Biotechnological techniques such as genetic transformation and RNA interference has allowed the silencing of gliadin genes, providing lines with very low gliadin content in the grains. We report a proteomic-based approach to characterize two low-gliadin transgenic wheat lines obtained by RNAi technology. These lines harbor the same silencing fragment but driven by two different endosperm specific promoters (γ-gliadin and D-hordein). The comprehensive proteome analysis of these transgenic lines by combining two-dimensional electrophoresis and RP-HPLC/nESI-MS/MS provided a large number of spots differentially expressed between the control and the transgenic lines. Hence, the results of this study will facilitate further safety evaluation of these transgenic lines for including in the diet of both gluten intolerant and/or wheat allergic patients.

Keywords: Celiac disease; Gluten proteins; Low-gliadins; Non gluten proteins; Transgenic wheat; Wheat allergy

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Wheat is among the three most cultivated cereal in the world. Despite its lower protein content (9-15%) compared with the starch (60-75%), proteins are very important for wheat functionality. Wheat grains are composed by two major groups of proteins: gluten proteins (gliadins and glutenins), responsible for the bread-making quality, and non-gluten proteins (metabolic and chloroform/methanol soluble proteins), with structural properties. Gliadins, responsible for the extensibility and viscosity of the dough, are monomeric and they are classified, according to its electrophoretic mobility in acid-polyacrylamide (A-PAGE), in α/β-, ω-, and γ-gliadins. On the other hand, glutenins comprise two major subunits: high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS), which provide elasticity to the dough. Nevertheless, the wheat proteins are associated with enteropathies and allergies such as allergy (0.2-0.5% population) (Zuidmeer et al.), non-celiac wheat sensitivity (NCWS) (6% population) (Sapone et al., 2011) and celiac disease (CD) (1% population) (Mariné et al., 2011). CD is an autoimmune disease provoked by the ingestion of wheat (gliadins), barley (hordeins), and rye (secalins), whose only treatment is a lifelong strict gluten free diet.

Wheat lines with silencing genes of α-gliadins (Becker et al., 2012), γ-gliadins (Gil-Humanes et al., 2008) and all gliadins (Gil-Humanes et al., 2010) have been developed by using interference RNA technology (RNAi). A comparative proteomic analysis was performed between two transgenic low-gliadin wheat lines obtained using RNAi and non-transformed wheat used as control. The aim of the analysis was to evaluate the silencing target of the genes and their possible effects on the accumulation of other wheat proteins. Because of the great complexity of the wheat proteins, the analysis of each protein fraction was performed separately: gliadins, glutenins, metabolic proteins and soluble protein in chloroform/methanol (CM-like), using two-dimensional electrophoresis followed by liquid chromatography-mass spectrometry (RP-HPLC/nESI-MS/MS). Comparisons were performed between pairs of genotypes namely BW208 *vs* D783, BW208 *vs* D793 and D783 *vs* D793, and among the three genotypes (BW208 *vs* D783 *vs* D793). The most important results are described below:

*Gliadins*

In the pairwise comparisons between wild-type and the transgenic lines (BW208 *vs* D783; BW208 *vs* D793), all spots down-accumulated in the transgenic lines were

identified as γ- and α/β-gliadins; spots over-accumulated in the line D783 corresponded to LMW-GS and none spots over-accumulated was found in the line D793. The comparison between both transgenic lines indicated that the line D793 presents a greater silencing of the gliadin genes, given that three spots denoted as γ- and α/β-gliadins were over-accumulated in the line D783.

*Glutenins*

Comparative analysis showed that all spots down-accumulated in transgenic lines corresponded to γ-gliadins (probably gliadin-like LMW-GS due to the high sequence similarity (Masci et al., 2002)), one uncharacterized protein and one heat shock protein. The over-accumulated spots in the transgenic lines were identified as HMW-GS DY10. None differences between both transgenic lines were found.

*Metabolic proteins*

The 2-DE analysis of metabolic proteins revealed significant differences between wild-type and each transgenic line. All spots over-accumulated in the transgenic lines belonged to serpins (serine protease inhibitors), an allergen group related to Bakers' asthma (Amano et al., 1998). The majority of down-accumulated spots in the transgenic lines were identified as β-amylases and only three serpins. The comparison between D783 and D793 showed one spot over-accumulated in the line D793 belonging to serpin.

*Chloroform/methanol soluble proteins*

The pairwise comparison with the wild-type showed that only one spot was down-accumulated in the line D783 (γ-gliadin) and two in the line D793 (γ-gliadin and granule-bound starch synthase). Almost all spots over-accumulated in both transgenic lines were identified as α-amylase/trypsin inhibitors (ATI) which are considered the main wheat protein related to baker's asthma (Armentia, 1994;Šotkovský et al., 2008). The remaining spots over-accumulated belonged to avenins and one heat shock protein. Although the number of spots over-accumulated for D793 was higher than for D783 when the comparison with the control was carried out, comparing both transgenic lines, only one spot was over-accumulated in the line D783 and it was identified as γ-gliadin.

In summary, these results shows that the strong silencing of gliadins have led to an increment in the HMW-GS, metabolic and chloroform/methanol soluble proteins, indicating that these lines would be suitable to celiac patients, controlling the daily intake since these lines still present toxic epitopes. However, these lines present greater amount of proteins related to respiratory allergy or bakers' asthma, therefore, these low-gliadin lines would not be advised.

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# *Chapter 3*

## Characterization of changes in gluten proteins in Low-Gliadin transgenic wheat lines in response to application of different nitrogen regimes

**Characterization of changes in gluten proteins in Low-Gliadin transgenic wheat lines in response to application of different nitrogen regimes**

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## Abstract

Gluten proteins are major determinants of the bread making quality of wheat but also of important gluten-related disorders. The gluten protein accumulation during grain filling is strongly influenced by nitrogen fertilization. We have characterized the gluten proteins in low-gliadin wheat lines as influenced by nitrogen treatments in two experiments. These transgenic lines, D783, D793, C655, D577 and E82, were obtained by using two different RNAi silencing fragments and two endosperm-specific promoters to drive the silencing fragments (D-hordein and γ-gliadin promoters). In Experiment 1 we used three nitrogen fertilizer rates (120, 360 and 1080 mg N) added at sowing stage and combined with two sulfur rates (8 and 30 mg S); Experiment 2 included two nitrogen levels (120 and 1080 mg N), which were added according to the greatest demand per plant using split applications. The protein quantification was accomplished by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and gluten content (ppm) determined using monoclonal antibody R5 (Competitive R5 ELISA). The results showed differences in protein accumulation between the two transgenic lines with the same silencing fragment but different promoter. Lines D793 and E82 showed low gliadin and an increment in glutenin content with increasing nitrogen. Competitive ELISA R5 showed a significant decrease in gluten content using split applications of nitrogen (Experiment 2) with 120 mg N compared to Experiment 1. In addition, line E82 ensures that variations in N fertilization will not result in increased gluten content.

Keywords: Transgenic wheat; promoters; gliadins; celiac disease; nitrogen fertilization; gluten; RP-HPLC; competitive R5 ELISA

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Wheat proteins are important for both technological properties of dough and also for human health. Wheat proteins are at lower proportion than starch, spite of that, they are considered the main source of proteins in several countries. These proteins are composed by gluten (gliadins and glutenins) and non-gluten proteins (albumins and globulins). The gliadins, monomeric fractions, comprise α/β-, γ-, and ω-gliadins and they are responsible for the extensibility and viscosity of the dough; whereas, the glutenins, with two types of subunits (high molecular weight and low molecular weight) provide elasticity to the dough. These gluten proteins are responsible for the final quality of bread and other baked products. Nevertheless, the wheat proteins are associated with enteropathies and allergies such as wheat allergy (0.2-0.5% population) (Zuidmeer et al.), non-celiac wheat sensitivity (NCWS) (6% population) (Sapone et al., 2011) and celiac disease (CD) (1% population) (Mariné et al., 2011). CD is an autoimmune disease provoked by the ingestion of gluten proteins from wheat, and their homolog proteins from barley (hordeins), and rye (secalins) (Trier, 1998). This disease is developed in genetically predisposed individual, being the individuals with genes encoding the human leukocyte antigen (HLA) DQ2 and -DQ8 those present higher risk. Hence, the only treatment for this disease is a lifelong strict gluten free diet (GFD), but it is very difficult since gluten is an additive widely used in the food industry and the risk of transgression in the diet is very high.

The development of biotechnology techniques like genetic transformation and interference RNA (RNAi), allowed silencing genes of α-gliadins (Becker et al., 2012), γ-gliadins (Gil-Humanes et al., 2008) and all gliadins (Gil-Humanes et al., 2010) present in wheat grains. The protein accumulation during grain filling is strongly influenced by nitrogen (N) fertilization (Wieser and Seilmeier, 1998). The fertilization treatments towards providing lines with reduced immunogenic characteristics are not well known. Adequate fertilization can produce modification on the grain protein content (López-Bellido et al., 1998;Wieser et al., 2004;Garrido-Lestache et al., 2005), according to that, using different concentrations of N and sulfur (S), both related in the activation/repression of the regulatory elements involved in the expression of prolamin proteins, we will be able to choose what nutrient balance is the adequate to obtain a reduction in gliadin content and a good yield.

Basing on that, gluten proteins were characterized in lines with a low-gliadin content subjected to different N concentrations and distinct fertilization strategies. Two

experiments were performed; in the Experiment 1, three N levels (120, 360 and 1080 mg N) were added at sowing together with two S levels (8 and 30 mg), and the lines used to quantify the proteins were D783, D793, C655, D577 (See Piston et al., 2013 for details) and BW208 (wild-type); in the Experiment 2, two levels of N were used (120 and 1080 mg N) which were added according to the time in which the demand for this nutrient was higher, using split applications. Lines used during this experiment were D783, D793, E82 and BW208 (See Piston et al., 2013 for details). Protein quantification was performed using the RP-HPLC, and the gluten content (ppm) was obtained using the monoclonal antibody R5 (ELISA competitive R5).

*Experiment 1*

Lines D783 and D793 (lines with a silencing fragment to target all gliadin fractions), significantly increased their HMW, LMW, total glutenins and total prolamins content with increasing N from 120 to 1080 mg; however, lines containing a silencing fragment to target only γ-gliadins (D577 and C655), all gliadins fractions increased with exception of γ-gliadins in the case of the line C655, without changes in the glutenins content. Moreover, the results showed that by adding the entire N at the sowing stage it produced an increment in the grain production but not in the kernel weight, indicating that the N available is directed to tiller production and more number of grains, according to (Oscarson, 2000). The ppms of gluten were determined by monoclonal antibody R5 in lines BW208, D783 and D793. The results indicated that there is a relationship between N and accumulation of toxic epitopes. In all three lines, gluten content increased with increasing N, but only for line BW208 this increment was significant.

*Experiment 2*

Wild type (BW208) and transgenic line D783 presented highly significant differences for all protein fractions with increasing N; however, in the line D793, α/β- and γ-gliadins were not modified but the remaining fractions increased significantly with 1080 mg N. The other line used in this experiment, the line E82, was only increased the ω-gliadin, HMW and total glutenins using 1080 mg N. Furthermore, our results showed that with split N fertilization the number of grains was not increased and the kernel weight significantly decreased. In this experiment, the gluten content (ppm) significantly increased with N supply in BW208, D783 and D793, and no changes in the line E82.

*Comparison between Experiments 1 and 2*

Providing 120 mg N, the gliadins, glutenins and total prolamins accumulation during Experiment 1 was twice of that for Experiment 2, and the gluten content (ppm) determined in Experiment 1 for BW208, D783 and D793 was 3.0, 2.5 and 1.3 times more than that for Experiment 2, respectively. However, with 1080 mg N, gliadin, glutenin and prolamin contents were similar in both experiments. This could indicate that split N and adding 120 mg N is desirable for a lower gluten content (ppm) to be provided. In both experiments, α/β- and γ-gliadins are not affected in D793 by increasing N, but glutenins increased, providing lower gluten content (ppm) than those determined for the line D783. Results obtained for the line E82 are of the greatest importance since total glutenins were increased without increasing the gluten content (ppm) when increasing N.

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# *Chapter 4*

## Effective identification of low-gliadin wheat lines by Near Infrared Spectroscopy (NIRS): implications for the development and analysis of foodstuffs suitable for celiac patients

PLOS ONE

RESEARCH ARTICLE

# Effective Identification of Low-Gliadin Wheat Lines by Near Infrared Spectroscopy (NIRS): Implications for the Development and Analysis of Foodstuffs Suitable for Celiac Patients

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## Abstract

### Scope

The aim of this work was to assess the ability of Near Infrared Spectroscopy (NIRS) to distinguish wheat lines with low gliadin content, obtained by RNA interference (RNAi), from non-transgenic wheat lines. The discriminant analysis was performed using both whole grain and flour. The transgenic sample set included 409 samples for whole grain sorting and 414 samples for flour experiments, while the non-transgenic set consisted of 126 and 156 samples for whole grain and flour, respectively.

### Methods and Results

Samples were scanned using a Foss-NIR Systems 6500 System II instrument. Discrimination models were developed using the entire spectral range (400–2500 nm) and ranges of 400–780 nm, 800–1098 nm and 1100–2500 nm, followed by analysis of means of partial least square (PLS). Two external validations were made, using samples from the years 2013 and 2014 and a minimum of 99% of the flour samples and 96% of the whole grain samples were classified correctly.

### Conclusions

The results demonstrate the ability of NIRS to successfully discriminate between wheat samples with low-gliadin content and wild types. These findings are important for the development and analysis of foodstuff for celiac disease (CD) patients to achieve better dietary composition and a reduction in disease incidence.

## Introduction

Wheat is the most widely cultivated cereal in the world due to its adaptability to different environments and high yields, as well as to the unique biomechanical properties of wheat dough.

Wheat is one of the most important crops in the world. The viscoelastic properties of the dough, due to its protein characteristics, are responsible for the quality of the final wheat products. These proteins are made up of gluten proteins and non-gluten proteins. Within gluten proteins are gliadins (α/β-, ω-, and γ-gliadins) and glutenins (HMW-GS and LMW-GS). These proteins in turn are responsible for a number of enteropathies, such as celiac disease (Mariné et al., 2011) and NWCS (Sapone et al., 2011), and allergies (Zuidmeer et al.). The development of varieties with a reduced toxic profile could help to enhance the diet of celiac people and reduce the incidence of CD as it was reported that the CD is related to the level and duration of gluten exposure (Ventura et al., 1999;Ivarsson et al., 2000). The development of biotechnology techniques as interference RNA (RNAi), allowed silencing genes of α-gliadins (Becker et al., 2012), γ-gliadins (Gil-Humanes et al., 2008) and all gliadins (Gil-Humanes et al., 2010) present in wheat grains.

A system able to distinguish between wheat lines non-genetically modified and wheat lines with low-gliadin content obtained with RNAi, is necessary to fast and effectively identification of those samples that could be used in the development of suitable products for celiac people. The Near Infrared Spectrometry (NIRS) is a technique that uses near-infrared region of the electromagnetic spectrum (800-2500 nm). The absorption bands are produced when NIR radiation vibrates as the same specific frequency (wavelength) as the molecular bonds of the analyzed sample, being the involved bonds: C-H, N-H and O-H. As a result, each sample has a unique profile, known as "footprint".

The discriminant analysis was performed in grains and flour. The transgenic samples included 409 samples as grains and 414 as flour, while the set of non-genetically modified samples was 126 and 156 for grain and flour, respectively. Besides, the calibration equation was validated twice using a set of samples non-used during calibration. The samples used for the first and second validation were from 2013 and 2014 multiplication years respectively.

We evaluated the different wavelength regions (800-1100nm, 1100-2500nm and the full spectrum). The wavelength region 800-1100nm do not obtained acceptable results. Also, we used different derivatives to obtain the best fit results. Using the samples from year 2013 to validate the calibration equation, the best validation model was obtained

when only the NIR region (1100-2500nm) and second derivative with scatter correction (SNV+D) were used. Thus, the classification error for whole grain was 0.7% and the validation error was 2.5%. In the case of flour, the classification and validation error was 1% and 2.5% respectively.

With the second validation samples, from year 2014), the best results were obtained using the full spectrum (400-2500nm). In the case of whole grain, the second derivative with scatter correction (SNV+D) obtained no samples misclassified during the calibration and a validation error equal to 3.8% given that seven samples were misclassified. For flour, the lowest percentage error (1.1%) was achieved with the first derivative and scatter correction (SNV+D) and only two samples were misclassified.

In this work were used low-gliadin transgenic lines with different plasmid combination (Gil-Humanes et al., 2010;Barro et al., 2016). Lines with lower toxicity corresponded with plasmid combinations 4, 5, 6 and 7 (See Barro et al., 2016 for details) and these lines, which have worthy prospects for commercialization in low-gluten foods, were correctly classified using the NIR technology.

The traceability in the commercialization of gluten free products is essential to ensure that those products do not suffer cross contamination and can be consumed by celiac patients and gluten sensitive people. We have demonstrated that the discrimination between lines with low-gliadin content and non-transgenic wheat, containing the full set of gliadins, is possible using the NIR technology, developing a robust model for the correct identification of those lines. Besides, the analysis was performed in grain and flour, having better results when the samples were in the form of flour, because the 99% of the flour samples and the 96% of the grain samples used during the validation, were correctly classified.

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# *Chapter 5*

## General conclusions and final remarks

# General conclusions and final remarks

## 5.1 General conclusions

1. Comparative proteome analysis demonstrated that transgenic lines D783 and D793, with strong down-regulation of gliadins by RNAi, presented an increment in the HMW-GS, metabolic and chloroform/methanol soluble proteins respect to wild-type, being line D793 the most affected by this proteomic variation.

2. Given that proteins of the α-amylase/trypsin inhibitor family, β-amylase and serpins, belonging to the metabolic proteins and chloroform/methanol soluble proteins (CM-like), are related to wheat allergens in food or respiratory allergy, the use of lines D783 and D793 by allergic people would not be advised, and therefore, assessments must be performed in relation to the different wheat related pathologies.

3. These low-gliadin lines would be suitable to people with a family history of gluten intolerance for which a decreased exposure to the triggering agent is desirable. However, the amount of food products made, potentially using the low-gliadin flour, should be quantified since such lines still present other gluten proteins in their composition.

4. Split N fertilization and adding 120 mg N (Experiment 2) is desirable for a lower gluten content (ppm), since adding the entire N at the sowing stage (Experiment 1) the gluten content (ppm) for BW208, D783 and D793 was 3.0, 2.5 and 1.3 times more than that in Experiment 2, respectively.

5. The results described in chapter 3 indicated that the promoter of line D793 (γ-gliadin) is more effective in the silencing of gliadins than that of D783 (D-hordein), resulting in a direct relationship with protein accumulation.

6. Transgenic lines D783 and E82 share the D-hordein promoter, but the effectiveness of the silencing is higher in line E82, indicating that it is required to combine the two silencing fragments (pDhpg8.1+pDhp_ω/α).

7. Line E82 was the best line for nitrogen fertilization, increasing N fertilization will not result in increased gluten content (ppm), and therefore it would be easier to manage by farmers.

8. The results presented on chapter 4 demonstrate the ability of NIRS to successfully discriminate between wheat samples with low-gliadin content and wild types, and results in flour were more reliable than in whole grain.

9. These findings are important for the development and analysis of foodstuff for celiac disease (CD) patients using these low-gliadin lines, to achieve better dietary composition and a reduction in disease incidence.

## 5.2 Final remarks

The development of wheat varieties with reduced immunogenic prolamin fractions may contribute not only to improving organoleptic and nutritional profile of the diet of CD patients and NWGS sufferers but may also be useful for anyone who decides to follow a gluten free diet. However, these lines are not recommended to allergic wheat people, due to the higher content in α-amylase/trypsin inhibitor family, β-amylase and serpins.

Nitrogen fertilizations strategies and amount of N applied influence on the accumulation of wheat storage proteins. The fact that with split N applications and 120 mg N is reduced the amount of ppm of gluten and that the line E82 does not undergo modifications in the ppm of gluten with variations of N, is important to minimize the accumulation of prolamins triggering CD but keeping a good bread making quality. Moreover, for the commercialization of gluten-free or low-gluten foods made using these low-gliadin lines, traceability is essential to ensure that these products do not suffer cross-contamination. Therefore, using NIR technology this control can be carried out.