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Analyses of albumins, globulins and amphiphilic proteins by proteomic approach give new insights on waxy wheat starch metabolism

Clément Debiton [a], Marielle Merlino [a], Christophe Chambon [b], Emmanuelle Bancel [a], Mélanie Decourteix [c], Véronique Planchot [d], Gérard Branlard [a,*]

[a] INRA UMR-1095, 234 avenue du Brézet, 63100 Clermont-Ferrand, France
[b] INRA UR-370 PFEM-Plateau Protéomique, 63122 Saint-Genès-Champanelle, France
[c] PIAF UMR-547, 24 avenue des Landais, 63177 Aubière, France
[d] INRA Unité Biopolymères Interactions Assemblages, 44316 Nantes, France

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ABSTRACT

Starch is composed of two types of glucose polymers: amylose and amylopectin. The *Waxy (Wx)* locus controls amylose synthesis in the wheat kernel. Hexaploid wheat has three *Wx* loci located on chromosomes 7A (*Wx-A1*), 4A (*Wx-B1*), and 7D (*Wx-D1*). Eight near isogenic lines (NILs) of *Triticum aestivum* cv. Tremie with one, two or three *Wx* null alleles were used. The albumin–globulin fraction, and amphiphilic proteins were separated using 2-dimensional electrophoresis (2DE) allowing the changes in the waxy kernel to be identified. Albumin–globulin fraction showed overexpression of sucrose synthases in the waxy NILs compared to the normal form of Tremie and a decrease in many proteins related to stress and defence metabolism such as serpins. A subunit of ADP-glucose pyrophosphorylase (AGPase), which is known to play a major role in starch synthesis, was also shown to be down regulated in the waxy NILs. Amphiphilic proteins confirmed the observations made on the albumin–globulin fraction with a decrease in a stress-related protein. These different regulations linked to observations made on wheat kernel (thousand kernel weight (TKW), protein amount per grain, size and distribution of the starch granules) led to formulation of the hypothesis that waxy endosperm does not reach maturity of the wild-type endosperm.

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1. Introduction

Wheat is the most widely-grown cereal in Europe and the second in the world after rice. Wheat starch is composed of approximately 28% amylose and 72% amylopectin, and is the major component of the grain representing 72% of grain weight (Buléon et al., 1998). In wheat grains, amylose content depends on the expression of the *waxy* genes: *Wx-A1*, *Wx-B1* and *Wx-D1*, which code for the granule bound starch synthase (GBSS) (Murai et al., 1999). These three genes are located on the short arm of chromosome 7A, the long arm of chromosome 4A, and the short arm of chromosome 7D respectively (Yamamori et al., 1994). Amylose free starches have been created by introgression of null forms for each of the three *waxy* alleles in hexaploid wheat (*Triticum aestivum* L.) (Nakamura et al., 1995) and their effect on starch composition and flour properties was reported (Yamamori and Quynh, 2000; Graybosch et al., 2003).

Understanding the mechanism involved in the synthesis of wheat starch granules still remains an important objective of wheat research and proteomic analysis of the amyloplast has been reported (Balmer et al., 2006; Dupont, 2008). Near isogenic lines of hexaploid waxy wheat for each of the three *waxy* null alleles (*Wx-A1b*, *Wx-B1b* and *Wx-D1b*) have been created in the agronomic cultivar Tremie using the Kanto 107 wheat, which lacks the Wx-A1 and Wx-B1 proteins, and Bai Huo partially waxy wheat, which lacks the Wx-D1 protein. The analysis of the response of the genome to the introduction of the three *waxy* null alleles may provide an opportunity to understand the mechanism associated with starch synthesis in wheat, notably amylose synthesis.

In the present study, proteomic analysis was used to analyze the albumin–globulin fraction and amphiphilic proteins in NILs with one, two or three *waxy* genes in the cultivar Tremie. These genetic responses have never been analyzed by proteomic analysis in the wheat endosperm. Most enzymes that occur in the endosperm belong to the albumin–globulin fraction or are amphiphilic proteins.

Abbreviations: 2DE, 2-Dimensional electrophoresis; AGPase, ADP-Glucose pyrophosphorylase; DArT, Diversity arrays technology; DW, Dry weight; GBSS, Granule bound starch synthase; NIL, Near isogenic lines; PVPP, Polyvinylpolypyrrolidone; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; Susy, Sucrose synthase; TKW, Thousand kernel weight; Wx, Waxy.

* Corresponding author. Tel.: +33 (0) 4 7362 4316; fax: +33 (0) 4 7362 4453.
E-mail address: branlard@clermont.inra.fr (G. Branlard).

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Several proteomic approaches have been successfully used to analyze the albumin–globulin proteins, which are water and salt–water soluble (Merlino et al., 2009; Vensel et al., 2005). This group of proteins is highly diversified due to their physicochemical properties in terms of amino acid composition, isoelectric point and molecular mass. They are involved in grain formation and in the accumulation of endosperm storage proteins and starch (Vensel et al., 2005). Amphiphilic proteins are detergent-soluble membrane proteins and were also studied using the same proteomic approach (Amiour et al., 2002). Most amphiphilic proteins are lipid-binding proteins and some of them are strongly linked to technological applications such as dough foaming properties. Proteomic analysis of these two groups of proteins was performed to understand the physiological response to the presence of *waxy* alleles compared to the normal form in the synthesis of endosperm starch. Albumin–globulin and amphiphilic proteins contain the major enzymes present in the grain and allow the description of the main events of the different metabolism pathways.

2. Experimental

2.1. Plant material

A waxy hexaploid wheat was created using the partially waxy mutant Kanto 107 (K107), which lacks the Wx-A1 and Wx-B1 proteins and Bai Huo, which lacks the Wx-D1 protein (Nakamura et al., 1995). These two hexaploid partially waxy wheats were used as progenitors to create eight NILs with null alleles at either one, two or three loci of the *waxy* genes in the French cultivar Tremie (Table 1). BC2 and BC3 were assisted with 36 SSR and 972 diversity arrays technology (DArT) markers (Pty Ltd, Yarralumla, Australia) to increase the isogenicity of each NIL with the remnant Tremie parent (see supplementary data Table A). The different NILs had reached from 96 to 99% of isogenicity. The BC3 lines were grown in soil in greenhouse conditions in 2008 with controlled temperature, day/night normal light, fertilizer plus full fungicide protection. Four plants for each NIL of Tremie were grown and harvested individually. We used mature grains of the BC3 self-pollinated NILs of Tremie for the three *waxy* null alleles to perform proteomic analyses of two protein fractions of the endosperm: the albumin–globulin fraction and amphiphilic proteins. The embryos were manually excised from the grain before the milling process. The absence of the waxy protein at either one, two or three loci was confirmed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) for the eight NILs (supplementary data, Fig. A).

2.2. Protein extraction

Albumin–globulin fraction and amphiphilic proteins were extracted three times for each of the eight NILs and two gel replicates were run for each extract. This way 48 2DE were made for the albumin–globulin fraction and 48 for the amphiphilic proteins.

Table 1
Name and allelic composition of the eight different isogenic lines for each of the three waxy null alleles in the French wheat cultivar Tremie.

	Name	Allelic composition		
		Wx-A1	Wx-B1	Wx-D1
Normal form	ABD	a (+)	a (+)	a (+)
Single null form	AB nD	a (+)	a (+)	b (−)
	AD nB	a (+)	b (−)	a (+)
	DB nA	b (−)	a (+)	a (+)
Double null form	A nBnD	a (+)	b (−)	b (−)
	B nAnD	b (−)	a (+)	b (−)
	D nAnB	b (−)	b (−)	a (+)
Triple null form	nAnBnD	b (−)	b (−)	b (−)

2.2.1. Albumins–globulins extraction

Albumins and globulins were extracted from 250 mg of whole-meal flour of wheat kernels whose embryos were hand removed. The kernels were ground in a Cyclotec® mill 1093 (Foss electric, Hilleröd, Denmark). Albumins and globulins were extracted in a saline solution (Phosphate 10 mM, NaCl 10 mM, pH 7.8, 4 °C) supplemented with a protease inhibitor cocktail for plant cell and tissue extracts P9599 (Sigma, St Louis, MO, USA). The supernatant containing albumins and globulins was separated from the pellet, which was retained for amphiphilic extraction. Albumins and globulins were precipitated with ice-cold acetone and the pellet was washed several times with acetone before drying.

2.2.2. Amphiphilic protein extraction

The amphiphiles were extracted from the pellet after extraction of the albumin–globulin fraction. The amphiphilic proteins were solubilized using Tris Triton X114 buffer (Tris-HCl 0.1 M, NaCl 0.25 M, EDTA 5 mM, Triton X114 2% (v/v), pH 7.8, 4 °C) supplemented with the antiprotease cocktail (Sigma, St Louis, MO, USA), according to Amiour et al. (2002). After stirring and centrifugation, the supernatant was warmed for 30 min at 37 °C for phase partitioning. The detergent rich phase was precipitated overnight in a solution of diethylether and ethanol 1/3 v/v at −20 °C. The precipitate was washed three times with 5 mL of diethylether/ethanol solution and twice with 5 mL of ice-cold diethylether to remove detergent, lipids and salts.

The different fractions were solubilized in a buffer (CHAPS 4% (w/v), Urea 7 M, Thiourea 2 M, IPG buffer pH 3–11 NL 1% (v/v) and DTT 70 mM), before isofocusing.

The protein concentration of the different fractions was measured using the Bradford protein assay (Sigma, St Louis, MO, USA).

2.3. Bi-dimensional electrophoresis

Twenty-four cm long immobiline strips (pH 3–11 NL) (GE Healthcare, Uppsala, Sweden) were first rehydrated overnight at room temperature with 450 µl of rehydration solution (CHAPS 4% (w/v), Urea 7 M, thiourea 2 M, IPG buffer (pH 3–11 NL) 1% (v/v), Destreak reagent (GE Healthcare, Uppsala, Sweden) 12 µl/mL). Protein extracts weighing 150 µg were deposited on the strip using cup loading with sample cups for manifold Ref:80649895 (GE Healthcare, Uppsala, Sweden), allowing a good separation without any streaking on the basic side of the 2D-gels. Isofocusing was carried out at 20 °C for 90 kV-hour with an Ettan IPGphor 2 unit (GE Healthcare, Uppsala, Sweden). After pH equilibration for 15 min in a solution of Urea 6 M, Tris-HCL 50 mM at pH 8.8, glycerol 30% (v/v) and SDS 2% (w/v) containing 1% of DTT (w/v), the proteins were alkylated for 15 min in the same solution containing 2.5% of iodoacetamide (w/v). The strips were deposited on SDS-Polyacrylamide gel with the following parameters ($T = 14\%$, $C = 2.1\%$) for albumin–globulin and amphiphilic fractions. Gels were stained overnight with colloidal Coomassie brilliant blue G250.

2.4. Image analysis

For each of the eight NILs, two replicates of three extracts were performed for the albumin–globulin and amphiphilic fractions. Gels were scanned using GS-800 scanner and Quantity one software (Biorad, Richmond, VA, USA). Images were analyzed using Samespot v3.2 (Nonlinear Dynamics, UK). Proteins were considered significantly different when the *p*-value of the ANOVA test was under 0.05, and when the q-value, which distinguished false

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