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RESEARCH ARTICLE

Genetic variation for waxy proteins and amylose content in Spanish spelt wheat (*Triticum spelta* L.)

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Abstract Spelt wheat is a neglected crop that could be used in the quality breeding of modern common wheat. One important aspect of this quality is the starch composition which is related to the waxy proteins. A collection of 420 accessions of Spanish spelt wheat was analysed for *waxy* protein composition by SDS–PAGE. Polymorphism was found in the three *waxy* proteins, detecting differences both in size and in activity, and a new *waxy* allele (*Wx-D1g*) was identified. Seed amylose content was also determined and significant differences were detected among the different allelic combinations. In general, the accessions carrying one or two *waxy* null alleles showed less amylose content. The variation found could be used to enlarge the genetic pool of common wheat, or to develop lines of spelt with different levels of amylose content.

Keywords Amylose content · Genetic resources · Starch · *Waxy* proteins

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Introduction

Starch is the main component of the wheat grain. Starch granules consist of two types of glucose polymers, the essentially linear amylose and the highly branched amylopectine, in a ratio in the range of 22–35%/68–75%. In addition, there are lipids and proteins linked to the starch granules although they are present in small quantities (Schofield and Greenwell 1987). The proteins associated with the wheat starch granules have been widely studied and these days their function is well known (Morell et al. 2001). The most important of these proteins, or at least the most studied, is the granule-bound starch synthase I (GBSSI) or *waxy* protein. This protein is the enzyme responsible for amylose synthesis in the seed. Since some starch properties such as gelatinization, pasting and gelation depend on the amylose/amylopectine ratio (Zeng et al. 1997), the *waxy* protein is crucial for flour quality.

Three *waxy* proteins are present in hexaploid wheats: *Wx-A1* (located on chromosome 7AS), *Wx-B1* (chromosome 4AL) and *Wx-D1* (chromosome 7DS) (Yamamori et al. 1994). Several studies have been carried out looking for polymorphism in wheat *waxy* proteins. The diversity found is low, even more when compared with other wheat seed proteins such as the storage proteins. Nonetheless, various forms of the *waxy* proteins have been found including *null* alleles, which are of particular interest. Nakamura et al. (1993) crossed two wheat cultivars lacking one and two different *waxy* proteins to get waxy wheat with amylose-free starch. This kind of

starch has been reported to be better for the noodles quality (Oda et al. 1980) and more efficient than standard wheat if the seed is used as substrate for biofuel production (Wu et al. 2006). Furthermore, resistant starch (high amylose content) is required by the food industry to create healthier foods, because resistant starch is less well digested in the small intestine than standard one (Topping and Clifton 2001). Therefore, the search for different forms of the *waxy* proteins is interesting in order to provide new resources for breeding programmes focused on starch properties.

One useful gene pool for wheat breeding is the hulled wheats (Padulosi et al. 1996). Our team has carried out several studies directed toward the evaluation of genetic resources of these underutilized and neglected wheats, mainly focussed on materials of Spanish origin. In the case of one of these, spelt (*Triticum spelta* (*T. aestivum* L. em. MacKey ssp. *spelta* (L.) Thell.), we have studied a large Spanish collection for the variation of morphological traits and seed storage proteins in hulled wheats (Caballero et al. 2001, 2004, 2008a, b; Alvarez et al. 2007). This species is still grown in Asturias (North of Spain), where, recently, a collecting mission was carried out to evaluate the existing genetic variability of this crop (Caballero et al. 2007).

The objective of the current study was to evaluate the polymorphism of the waxy proteins, as well as its possible effects on the amylose content, in the same Spanish spelt wheat collection previously evaluated for morphological and seed storage proteins content by our team.

Materials and methods

Plant materials

Four hundred and twenty accessions of Spanish spelt wheat obtained from the National Small Grain Collection (Aberdeen, USA), Centre for Genetic Resources (Wageningen, Netherlands) and the Plant Genetic Resource Center-INIA (Centro de Recursos Fitogenéticos-INIA, Alcalá de Henares, Spain), were analysed in this study. Most of these accessions are landraces and were collected in Asturias (province in the north of Spain). These plants were grown during 2006–2007 and 2007–2008 in the IFAPA station (Cordoba, Spain) according to standard agricultural practice in the region. Several spikes per plant were protected to avoid random crosses. Chinese Spring and Mexicali cultivars were used as standards.

Starch extraction and electrophoretic analysis

Twenty milligrams of flour were mixed with 1 ml of distilled water and incubated at 4°C for 24 h. The homogenate was filtered through Miracloth and centrifuged at 14,000 g for 1.5 min. The pellet was washed with 1 ml of buffer A [55 mM Tris–HCl pH 6.8, 2.3% (w/v) sodium dodecyl sulphate, 2% (w/v) dithiotreitol, 10% (v/v) glycerol], according to Echt and Schwartz (1981). Then, 1 ml of buffer A was added to the pellet and was left for 30 min at room temperature. The pellet was washed three times with distilled water, once with acetone and then air-dried. The residue was mixed with 80 µl of buffer A, heated in a boiling bath for 2 min, cooled in ice and centrifuged.

An aliquot of supernatant (20 µl) was loaded in vertical SDS–PAGE slabs in a discontinuous Tris–HCl-SDS buffer system (pH: 6.8/8.8) at a polyacrylamide concentration of 12% (w/v, $C = 0.44\%$). The Tris–HCl/glycine buffer system of Laemmli (1970) was used. Electrophoresis was performed at a constant current of 30 mA/gel at 18°C for 4 h after the tracking dye migrated off the gel. Protein bands were visualised by silver staining.

Amylose analysis

All samples for each waxy combination found were evaluated for apparent amylose content (Table 2). This content was determined in triplicate as described by Watanabe et al. (1998).

Statistical analyses

Allelic frequency and the effective numbers of alleles per locus (*Ne*) at the *Wx-A1*, *Wx-B1* and *Wx-D1* loci were calculated. Genetic diversities was estimated using Nei's diversity index -*He*- (Nei 1972, 1973).

Data on amylose content were analysed by ANOVA, and means were compared using Duncan's multiple range test.

Results

Polymorphism at GBSSI or waxy proteins

The electrophoretic analysis of the *waxy* proteins revealed polymorphism for the three *waxy* loci (Fig. 1; Table 1). For the *Wx-A1* locus, two alleles were found: the *Wx-A1a*, similar to the allele present in cv. Chinese Spring, and the *Wx-A1b* (*null* type), which lacks the protein (lanes 1, 2 and 5). The *Wx-A1b* allele was present in 36 accessions (8.57%).

For the *Wx-B1* locus, three different alleles were found: the *Wx-B1a* as Chinese Spring (lane 6); the *null* protein *Wx-B1b* (lane 3); and the *Wx-B1c′* allele, with less mobility than *Wx-B1a* (lane 7). Both *Wx-B1b* and *Wx-B1c′* alleles were less common (11.67 and 12.86%).

The lines evaluated presented a large homogeneity for the *Wx-D1* locus, where 99.52% of the accessions showed the *Wx-D1a* allele. Two additional alleles were detected: the *null* allele (*Wx-D1b*, lane 4) that was only present in one accession; and a novel *waxy* allele (lane 5), not previously described, with a slightly lower electrophoretic mobility than the *Wx-D1a* allele. This new allele was provisionally named *Wx-D1g*.

The effective number of alleles (*Ne*) was especially low for the *Wx-D1* locus, where one of the detected alleles (*Wx-D1a*) was clearly dominant over the others. This indicates that the other two of the alleles detected are in clear danger of erosion. In addition, the *He* value for this locus was very low (Table 1), representing approximately 10% of the genetic diversity that this locus would have if the allelic variants were distributed randomly.

Table 1 Allelic frequencies and genetic parameters at three waxy loci in 420 Spanish spelt wheat accessions

Locus	Allele	Accessions		*Ne*	*He*
		N	%		
Wx-A1	a	384	91.43	1.186	0.157
	b	36	8.57		
Wx-B1	a	317	75.48	1.667	0.400
	b	49	11.67		
	c′	54	12.86		
Wx-D1	a	418	99.52	1.010	0.009
	b	1	0.24		
	g	1	0.24		

Ne effective number of alleles, *He* genetic diversity

Considering the variation for the *Wx-A1*, *Wx-B1* and *Wx-D1* loci, eight combinations were detected. Their frequencies are shown in Table 2, where the clear dominance of the combination *Wx-A1a*, *Wx-B1a*, *Wx-D1a* can be observed; it appears in 69.52% of the samples. For the rest, three combinations appeared with a relatively high frequency, the combination *Wx-A1a, Wx-B1c′, Wx-D1a* appears in 47 accessions, *Wx-A1a, Wx-B1b, Wx-D1a* in 44 and *Wx-A1b, Wx-B1a, Wx-D1a* in 23. The other four combinations were rare with frequencies of under 5% (Table 2).

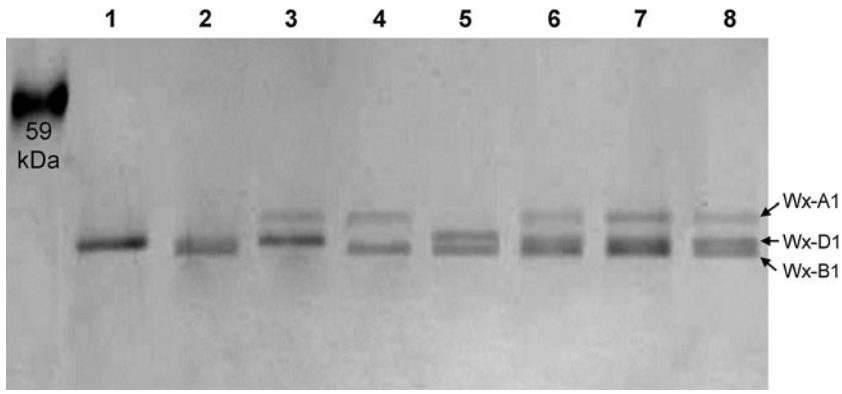

Fig. 1 SDS–PAGE gel electrophoresis patterns of *waxy* proteins. *Lanes* are as follow: **1,** PI-348544 (*Wx-A1b, Wx-B1b, Wx-D1a,*); **2,** BGE-012911 (*Wx-A1b*, *Wx-B1a, Wx-D1a*); **3,** BGE-012913 (*Wx-A1a, Wx-B1b, Wx-D1a*); **4,** PI-348693 (*Wx-A1a, Wx-B1a, Wx-D1b*); **5,** PI-348701 (*Wx-A1b, Wx-B1a, Wx-D1g*); **6,** cv. Chinese Spring (*Wx-A1a, Wx-B1a, Wx-D1a*); **7,** PI-348495 (*Wx-A1a*, *Wx-B1c′, Wx-D1a,*); and **8,** cv. Chinese Spring (*Wx-A1a, Wx-B1a, Wx-D1a*)

Table 2 Allelic composition and apparent amylose content (%) at the three *waxy* loci and number of the accessions with different *waxy* alleles

Allelic composition	*N*	%	Accession standard	Amylose content % (SE)[a]
Wx-A1a, Wx-B1a, Wx-D1a	292	69.52	BGE-001997	41.61b (1.61)
Wx-A1b, Wx-B1a, Wx-D1a	23	5.48	BGE-012911	39.65c (1.28)
Wx-A1a, Wx-B1b, Wx-D1a	44	10.48	BGE-012913	38.89cd (1.37)
Wx-A1a, Wx-B1c′, Wx-D1a	47	11.19	PI-348495	42.99a (1.54)
Wx-A1b, Wx-B1c′, Wx-D1a	7	1.67	BGE-012939	39.45c (2.11)
Wx-A1a, Wx-B1a, Wx-D1b	1	0.24	PI-348693	38.93cd (1.55)
Wx-A1b, Wx-B1a, Wx-D1g	1	0.24	PI-348701	40.14c (3.61)
Wx-A1b, Wx-B1b, Wx-D1a	5	1.19	PI-348544	37.27d (2.09)

[a] SE standard error; means with the same letter are not significant different at 95%

Apparent amylose content

Apparent amylose content was measured in all accessions evaluated, which were grouped according to *waxy* allelic combination (Table 2). An analysis of variance was carried out using the allelic combination as variation source. The differences between accessions per each combination were not significant, while the differences between combinations were significant. The results from comparison of means by the Duncan's multiple range test is shown in Table 2. In general, higher values were found in the accessions with the three active *waxy* alleles, while the accessions carrying one or two *null waxy* alleles had lower values.

Discussion

In this study, a wide collection of Spanish spelt wheat was analysed to study polymorphism in the *waxy* proteins. This collection represents a substantial proportion of the Spanish spelt wheat accessions conserved in Germplasm Banks.

Although the polymorphism found was low, different forms for each *waxy* protein (*Wx-A1, Wx-B1* and *Wx-D1*) were detected. *Null* forms of each locus were detected. These are very important for breeding programmes focused on the development of *waxy* wheats (amylose-free starch). Several accessions contained the *Wx-A1b* (8.75%) or *Wx-B1b* (11.63%) proteins, these results being similar to those found by Rodriguez-Quijano et al. (1998) in a similar study focused on *T. aestivum* and *T. spelta* Spanish wheats *(Wx-A1b,* 7.63%; *Wx-B1b,* 12.50%). The detection of the *null* allele for the *Wx-D1* locus is particularly important. This allele is extremely rare and has not been described previously in spelt wheat. As far as we know this *null* allele has been detected previously only in five accessions of common wheat (Yamamori et al. 1994; Urbano et al. 2002). As cv. Bai Huo (*Wx-D1b)* is repeatedly used in breeding programmes, the discovery of new materials carrying the *Wx-D1 null* allele is important in order to enlarge the range of possible parentals used in *waxy* wheat production.

In the cases of the *Wx-B1* and *Wx-D1* proteins differences in size were also detected between the different accessions. One of the alleles found (*Wx-D1g)* has not been described previously and was only present in one line. Another well-known allele, *Wx-B1c′*, was found in several accessions, with a similar frequency (12.82%) to that described by Yamamori et al. (1994) in their study with *T. aestivum* accessions from all over the world (15.70%) and higher than that described by Rodriguez-Quijano et al. (1998) in a study on Spanish spelt (3.47%).

Apparent amylose content was determined in samples of each allelic combination of *waxy* loci detected in order to determine whether the differences found affect the amylose content. Although the method used overestimates the amylose values it seems very useful to compare the different genotypes. Significant differences were found between some allelic combinations; in particular it seems evident that samples carrying one or two null *waxy* alleles have less amylose than those carrying the three *waxy* proteins, which is in agreement with the results of Yamamori and Quynh (2000). These authors also established that the lack of *Wx-A1* protein had less effect than the lack of *Wx-B1* or *Wx-D1* proteins. Although the differences between accessions *Wx-A1* null and *Wx-D1* null or *Wx-B1 null* were not

significant, the accessions lacking *Wx-D1* protein or *Wx-B1* protein showed lower mean values, supporting the data from Yamamori and Quynh (2000), who found that *Wx-B1* protein is the protein which generates most amylose and *Wx-D1* the second one.

In conclusion, the current study has demonstrated that the safeguard of these spelt accessions, together with others stored in Germplasm Banks, is fundamental for the maintenance of the genetic diversity in this crop. This diversity could be of special relevance for plant breeding, mainly for starch quality. In addition, spelt is an interesting genetic resource for the search of useful genes for plant breeding of the modern wheats, and could enlarge the gene pool of *waxy* proteins in common wheat.

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