Journal of Cereal Science 52 (2010) 187–191

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

A novel enzyme-linked immunosorbent assay for the detection of *Wx-B1* null wheat lines using a monoclonal antibody and its application for the detection of marker heterogeneity within commercial cultivars

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ARTICLE INFO

Article history:
Received 18 December 2009
Received in revised form
7 April 2010
Accepted 6 May 2010

Keywords:
Granule bound starch synthase (GBSS)
Waxy
Enzyme-linked immunosorbent assay (ELISA)
PCR

ABSTRACT

A monoclonal antibody was raised with specificity for the granule bound starch synthase (GBSS1) *Wx-B1* homeoallele of wheat (*Triticum aestivum* L.) using a synthetic peptide immunogen. This was used to develop a simplified enzyme-linked immunosorbent assay (ELISA) for the discrimination of *Wx-B1a* and *Wx-B1b* alleles which differ in the starch properties they confer. Discrimination of these alleles is important for the selection of wheat lines for Udon-style noodle production. The simplified ELISA worked in a single antibody (indirect) format and gave improved ease of use, discrimination of alleles and resolution relative to a previously developed 2-antibody (sandwich) ELISA. When the test was validated using breeders seed of a panel of commercial cultivars, heterogeneity of *Wx-B1* alleles was observed for a significant proportion of the cultivars tested and this was confirmed using PCR analysis of the *Wx-B1* and *Wx-A1* genes. This observation has implications for cultivar wheat quality assessment, the application of molecular markers for variety identification purposes and the establishment of mapping populations from commercial cultivars.

1. Introduction

In wheat, (*Triticum aestivum* L.) granule bound starch synthase (GBSS) comprises two distinct types, GBSS1 and GBSS2 (MacDonald and Priess, 1985; Mason-Gamer et al., 1998). GBSS1 is responsible for the synthesis of amylose in the developing endosperm and pollen, and is encoded by three genes, present at the *Wx-A1* locus on the short arm of chromosome 7A, the *Wx-B1* locus on the long arm of chromosome 4A, and the *Wx-D1* locus located on the short arm of chromosome 7D (Ainsworth et al., 1993). Wheat lines lacking the *Wx-B1* locus are common in Australia and are designated as *Wx-B1b* (gene deleted and Wx-B1 protein absent) in contrast to *Wx-B1a* lines (*Wx-B1* protein present).

Wx-B1b lines have altered starch properties, including decreased amylose content and increased flour swelling power, and are strongly favoured for the development of cultivars for the production of white salted (Udon style) noodles (Zhao et al., 1998). Graybosch et al. (1999) reported the use of a sandwich enzyme-linked immunosorbent assay (ELISA) for the quantification of total GBSS1 in wheat using monoclonal antibodies, but without specific affinity for any one isoform. Subsequently, Gale et al. (2004) developed a sandwich ELISA based on a non-isoform-specific monoclonal antibody and a *Wx-B1* isoform-specific anti-synthetic peptide polyclonal antibody for use as a screening tool in wheat breeding programs (Gale et al., 2001). Here we report the development of a monoclonal antibody with specificity for the *Wx-B1* isoform of GBSS1 and the development of a novel, single antibody ELISA for discrimination of *Wx-B1a* and *Wx-B1b* wheat lines. The test is simple to perform, and can be adapted easily to screen large numbers of samples.

2. Experimental

2.1. Seed stocks

Seed samples were obtained from the Australian Winter Cereals Collection (AWCC), Tamworth, Australia.

2.2. Monoclonal antibody development

The GBSS1 *Wx-B1* isoform-specific synthetic peptide Wx-B1.2 (EAPRILDLNNNPYFGC-MBS) linked to keyhole limpet haemocyanin using MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester) cross-linker (Pierc-Rockford, IL. USA) was used for the immunization of female BaLB/C mice according to standard immunization procedures (Skerritt et al., 1984). Initial immunisations were of 300 μg of

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doi:10.1016/j.jcs.2010.05.002

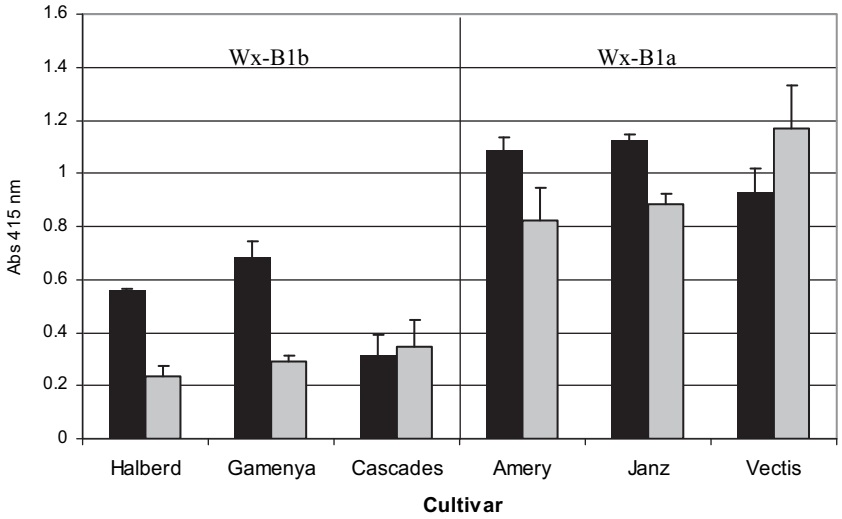

Fig. 1. Comparison of sandwich and indirect ELISA. Results are average of two wells, with reagent blank value subtracted; error bars represent 1 SD. Key: ■ PAb Sandwich ELISA, antigen dilution 1/20, ▒ MAb Indirect ELISA, antigen dilution 1/5.

peptide-carrier protein conjugate, injected subcutaneously and intramuscularly, with three additional injections of 150 μg over a six-week period. Hybridomas were produced (Skerritt et al., 1984) and positive wells (165) were screened using an indirect ELISA format (detailed below) for detection of antibodies that are bound to extracted GBSS protein from either cultivar Rosella (*Wx-B1b*) or Suneca (*Wx-B1a*). One monoclonal line (14C6) that displayed differential signal between the two wheat cultivars was subject to limiting dilution to produce clonal lines (72), of which 17 showed specificity for the *Wx-B1* isoform of GBSS1. The isotype of one selected *Wx-B1*-specific hybridoma (14C6-5B5) was determined to be IgG1 (λ light chain) using the Isostrip – Mouse Monoclonal Antibody Isotyping Kit (Roche, Indianapolis, USA).

2.3. Antigen extraction

GBSS1-containing wheat extracts were prepared as follows. Half or whole grains were placed into 1 mL microtitre tubes (Trace plastics – Thermo-Trace, Melbourne Australia) and soaked in 200 μL of water overnight at room temperature to soften. One 5 mm stainless steel ball bearing was added to each tube, which was shaken at high speed using a Cap Mix (ESPE GmbH & Co. KG, FRG), for 2 s. Urea (Sigma Chemicals, 700 μL of a 10 M solution) was added to each sample and the tubes gently mixed end-over-end for 30 min. The homogenates were cleared by centrifugation at 850 RCF (Sigma model 4K15) for five minutes and the supernatants diluted and used for ELISA analysis.

2.4. ELISA protocol

Urea-extracted antigen (diluted 1:5 in 50 mM carbonate buffer, pH 9.5) was coated onto microtitre plate wells (100 μL/well, in duplicate wells) overnight. Plates were washed 3 times with phosphate-buffered saline-0.05% Tween 20 (PBS-T) and blocked by incubation with PBS-1% bovine serum albumin (BSA, Sigma, 150 μL/well) for 60 min. Plates were emptied, tapped out onto dry towelling and MAb 14C6 1B5 added (10 μg/mL, 100 μL per well) followed by incubation for 60 min. Plates were washed 3 times with PBS-T to remove unbound antibody. A commercial HRP-labelled rabbit anti-mouse IgG (Dako, Glostrup, Denmark) was added (150 μL per well of a 1/1500 dilution) and incubated for 30 min. Plates were washed 3 times with PBS-T and developed by the addition of 150 μL of substrate (2 mM 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid (ABTS, Sigma) in 0.1 M sodium citrate buffer, pH 4.5, containing 0.003% (v/v) H_2O_2). After 60 min, absorbance values were measured at 415 nm using a V Max plate reader (Molecular Devices, Sunnyvale, CA, USA). All steps were performed at room temperature.

2.5. Multiplexed GBSS1 homeoallele specific PCR

Primers with specificity for either the *Wx-B1* or *Wx-A1* homeoallele of GBSS1 were designed. Multiplexing permitted the use of the *Wx-A1* amplicon as a positive control for discrimination of the *Wx-B1b* allele where the gene is deleted, from the functional *Wx-B1a* allele. Primer sequences (5′ to 3′) were: *Wx-B1F*: CCCCGAAGCAACAAAGCCG, Wx-B1R: GACCGTTGGCCTGCAGAC, *Wx-A1F*: GGTTCTGGGTGGATTCTG and Wx-A1R: AGCGGCTGGTGACCGCC.

DNA samples were prepared from half grains using a simple high throughput method Half seeds were placed in 1 mL microtitre tubes (Trace Plastics-Thermo –Trace) and one 5 mm steel ball added, 200ul of extraction buffer (200 mM Tris HCL pH 8.0, 250 mM NaCl, 25 mM EDTA pH 8.0) was added and samples shaken on a vibrating Ball Mill (MM 300 Retsch Germany) for 3 min. Samples were centrifuged at 700 g and 90 μL of supernatant was removed and placed into a fresh well to which 10 μl of 5% SDS (w/v) was added. These were incubated at 65 °C for 60 min before centrifugation for 10 min at 2200 g, 40 μl of supernatant was removed into a fresh well containing 100 μl of ethanol and mixed gently. Samples were again spun at 700 g for 10 min, the supernatant removed and the pellet air dried. The pellet was resuspended in 100 μl of 0.1 × TE Buffer, centrifuged at 700 g for 5 min.

PCR was performed in a reaction volume of 20 μL, containing 50 ng DNA, 2 units Hotstar Taq DNA polymerase (Qiagen), 1 × PCR buffer (Qiagen, 1.5 mM $MgCl_2$), 200 μM dNTPs and 10 pmole of each of the 4 primers. Cycling conditions were 94 °C for 5 min, followed by 38 cycles of 94 °C for 30 s, 55 °C for 40 s, 72 °C for 30 s. Samples were separated on 2% agarose (Progen Industries) gels (120 V, 1.5 h) and visualised using ethidium bromide.

3. Results and discussion

Preliminary analysis of three *Wx-B1a* and three *Wx-B1b* wheat cultivars was conducted using an indirect (single antibody) ELISA as