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Genetic control of amylose content in wheat endosperm starch and differential effects of three *Wx* genes

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Abstract The endosperm starch of the wheat grain is composed of amylose and amylopectin. Genetic manipulation of the ratio of amylose to amylopectin or the amylose content could bring about improved texture and quality of wheat flour. The chromosomal locations of genes affecting amylose content were investigated using a monosomic series of Chinese Spring (CS) and a set of Cheyenne (CNN) chromosome substitution lines in the CS genetic background. Trials over three seasons revealed that a decrease in amylose content occurred in monosomic 4A and an increase in monosomic 7B. Allelic variation between CS and CNN was suggested for the genes on chromosomes 4A and 7B. To examine the effects of three *Waxy* (*Wx*) genes which encode a granule-bound starch synthase (Wx protein), the Wx proteins from CS monosomics of interest were analyzed using SDS-PAGE. The amount of the Wx protein coded by the *Wx-B1* gene on chromosome arm 4AL was reduced in monosomic 4A, and thus accounted for its decreased amylose content. The amounts of two other Wx proteins coded by the *Wx-A1* and *Wx-D1* genes on chromosome arms 7AS and 7DS, respectively, showed low levels of protein in the monosomics but no effect on amylose content. The effect of chromosome 7B on the level of amylose suggested the presence of a regulator gene which suppresses the activities of the *Wx* genes.

Key words Amylose content · Monosomic · Substitution line · *Triticum aestivum* · *Wx* protein

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Introduction

The endosperm texture of the wheat grain is one of the primary determinants of manufactured products, because it affects the quality of resultant flours as well as milling performance. In countries such as Japan and China, the flour from soft wheats is often processed into noodles. Both quantitative and qualitative aspects of the starch deposited in the endosperm play an important role in the processing and eating qualities of noodles (Moss 1979; Oda et al. 1980; Toyokawa et al. 1989). Wheat endosperm starch usually consists of two different forms of polymer, amylose and amylopectin, and the ratio of amylose to amylopectin or the amylose content varies between cultivars (Kuroda et al. 1989). Low-amylose-content cultivars have been prefered for noodle manufacture (Miura and Tanii 1994). Therefore, genetic manipulation of amylose content may be expected to contribute to the improved texture and quality of the flour.

In maize and rice, it is reported that the production of amylose in the endosperm is controlled by the *Waxy* (*Wx*) locus (Echt and Schwartz 1981; Sano 1984). *Wx* genes on chromosome 9 of maize and those on chromosome 6 of rice encode for a granule-bound starch synthase of about 60 kDa known as the Waxy (Wx) protein. The amount of Wx protein is related to amylose content in the endosperm starch of rice (Sano 1985) and maize (Imam 1989).

The 60-kDa Wx protein is also produced in wheat endosperm starch (Schofield and Greenwell 1987; Yamamori et al. 1992). Chao et al. (1989) identified three *Wx* genes in Chinese Spring wheat. These genes are organized as a triplicate set of single-copy homoeoloci on chromosome arms 7AS, 7DS and 4AL, which carries a translocation of a segment from 7BS (Naranjo et al. 1987; Liu et al. 1992). Nakamura et al. (1993a), using two-dimensional polyacrylamide-gel electrophoresis, separated the *Wx* gene products into three distinct subunit groups of Wx proteins, which were encoded by the

Wx-A1, *Wx-B1* and *Wx-D1* genes on Chinese Spring chromosome arms 7AS, 4AL and 7DS, respectively. The total amount of *Wx* protein correlated with the amylose content (Yamamori et al. 1992; Miura and Tanii 1994), so it is of interest to determine whether different amounts of particular subunit groups of Wx proteins are responsible for the differences in the level of amylose. In this article we report on the chromosomal locations of genes affecting amylose content and the differential effects of the three *Wx* genes.

Materials and methods

Plant materials

The monosomic series of Chinese Spring (CS), developed by Sears (1954), and the Cheyenne (CNN) chromosome substitution lines into CS, developed by Morris et al. (1966), were used to identify the chromosomal control of amylose content. The chromosome nomenclature used here is that agreed at the 7th International Wheat Genetics Symposium, where the previous designations of 4A and 4B were reversed.

Preparation of grain samples

The CS monosomics and CS (CNN) substitutions were grown at the research field of Obihiro University, Obihiro, Japan. The CS monosomics were grown together with the euploid in spring-sown trials for three seasons, 1990–1992. The CS (CNN) substitutions were grown together with their parents from autumn sowings in the 1990/91 and 1991/92 seasons, and from a spring sowing in the 1990 trial, where seedlings were vernalized beforehand. More than ten plants per line were grown and harvested in August for all trials.

Monosomic plants were selected cytologically by counting the chromosome number at metaphase in squash preparations of root-tip cells taken from germinated grains, pre-treated with ice, and stained with Schiff's reagent.

Measurement of amylose content

One hundred grams of grain per line were milled on a Brabender Quadrant Junior Test Mill to a final extraction rate of 60%. Starch granules were separated using conventional methods. One hundred milligrams of starch granules were weighed accurately and the percentage amylose content was colorimetrically determined with iodine using the Auto Analyzer System II (Bran + Lubbe Co.). In this system, amylose content was estimated by comparison to artificially-synthesized standards. Each assay was carried out at least twice.

Student's t-test was performed for one-season data to detect significant differences between parental controls and each of the monosomic or substitution lines. For the three-season data, analysis of variance was performed and the line × season interaction mean square was used as an estimate of experimental error.

Electrophoresis

The expression of Wx proteins was assessed in the CS monosomics and CS (CNN) substitution lines for chromosome 4A and the three members of homoeologous group 7. The Wx proteins extracted from the CNN monosomics for these four chromosomes, developed by R. Morris (University of Nebraska), were also examined. The selection of monosomics was carried out as previously described where embryo half grains were germinated and used to determine chromosome number.

The endosperm half of monosomic grains was crushed and starch granules were isolated according to the method described by Echt and Schwartz (1981). An aliquot of 5 mg of the granules was mixed with 70 µl of SDS-buffer (0.055 *M* Tris-HCl, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol) and heated for 5 min in a boiling water bath; then the gelatinized solutions were cooled on ice and centrifuged at 15 000 rpm for 5 min. The supernatants were analyzed by sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE).

The Wx proteins were first separated into high-molecular-weight (HMW) and low-molecular-weight (LMW) proteins by Laemmli's SDS-PAGE system (Laemmli 1970) with one minor modification; a 10% separating gel with an acrylamide/bis acrylamide ratio of 30:0.135 instead of 30:0.8. Proteins were stained with silver stain kits (Wako Pure Chemical Industry Ltd.).

The LMW *Wx* proteins were then characterised by two-dimensional polyacrylamide-gel electrophoresis (2D-PAGE) as described by Nakamura et al. (1993 b). An aliquot of 10 mg of starch granules was mixed with 400 µl of lysis buffer containing 8 M urea, 2% (v/v) Nonidet-P40, 2% (v/v) ampholine, pH 3.5–10, 5% (v/v) 2-mercaptoethanol, and 5% (w/v) polyvinyl-pyrrolidone. The solution was boiled for 1 min and immediately cooled on ice; the slurry was then centrifuged at 15 000 rpm for 10 min at 4 °C. The supernatants were used for isoelectric focusing (IEF) as the first dimension of electrophoresis. The SDS-PAGE system with a 15% separating gel in the ratio of 30:0.135 acrylamide/bis acrylamide was employed for the second dimension. Proteins were stained with silver stain.

Results

Amylose content

The variation in amylose content obtained from studies of the CS monosomic is given in Table 1. The mean amylose content of CS across three seasons was 24.27% (24.27 mg per 100 mg of starch granules) and its standard

Table 1 The mean deviations for amylose content of the CS monosomics from the CS euploid, grown in the spring-sown trials for three seasons, 1990–1992

Chromosome	Deviations from CS			Mean
	1990	1991	1992	
1A	−0.36	0.64	0.77*	0.35
1B	0.28	−0.63	1.07**	0.24
1D	0.50	0.04	0.43	0.32
2A	−0.26	−0.52	−0.30	−0.36
2B	−0.81	0.17	−0.09	−0.24
2D	−0.43	−0.82	−0.52	−0.59
3A	−0.18	0.51	0.14	0.16
3B	−0.48	−0.67	0.10	−0.35
3D	0.23	−0.37	−0.30	−0.15
4A	−2.24**	−1.97**	−0.83**	−1.68***
4B	−0.58	−1.25	0.22	−0.54
4D	0.27	−1.36*	0.14	−0.32
5A	0.20	−0.62	0.00	−0.14
5B	0.25	0.17	−0.19	0.08
5D	0.03	−0.07	0.38	0.11
6A	−0.05	0.17	0.22	0.11
6B	−0.03	−0.49	−0.01	−0.18
6D	−0.50	−0.76	0.14	−0.37
7A	0.25	0.11	0.62	0.33
7B	1.21**	0.88*	0.86**	0.98**
7D	−0.55	0.11	−0.02	−0.15
CS	24.50	24.75	23.56	24.27

*,**,*** Significantly different from the CS euploid at 5%, 1% and 0.1% levels, respectively

deviation between seasons was less than 1%. The mean deviation across seasons in each CS monosomic line from the euploid was considered as an estimate of the individual effects of CS chromosomes on amylose content. The hemizygous condition of chromosomes 4A and 7B gave significant effects. Monosomic 4A produced grains with a 1.68% lower amylose content than the grains in CS, whereas monosomic 7B yielded about a 1% higher content than CS in every season. Monosomic lines of chromosomes 1A and 1B also produced grains with increased amylose content in the 1992 season.

Table 2 shows that the substitution of CS chromosome 7B by its homologue from CNN reduced the amylose content by 1.25%. The 4A homologue of CNN gave an increased content in the first two seasons, while its effect could not be observed in the third season. Significant deviations from CS were found for substitutions involving CNN chromosomes 1A, 2D and 7A but were limited to only one season.

It was evident from these results that the major effects on amylose content were confined to chromosomes 4A and 7B, while additional minor effects were associated with several chromosomes including those of homoeologous group 1.

Wx proteins

The SDS-PAGE electrophotograms of Wx proteins extracted from the endosperms of CS and four monosomic lines for chromosome 4A and the members of homoeologous group 7 are presented in Fig. 1. The Wx proteins from CS and monosomics 4A, 7B and 7D clearly separated into HMW and LMW proteins but it was hard to detect any differences between CS and these monosomic lines. Monosomic 7A was however distinguishable from CS by a greatly reduced HMW.

Using the 2D-PAGE system, the LMW Wx proteins from CS and monosomics 4A, 7A, 7B and 7D were analyzed further. The banding pattern of CS, illustrated in Fig. 2, shows that the HMW and LMW Wx proteins are composed of a complex with several subunits. According to the classification of Nakamura et al. (1993 a), simplified in Fig. 3, three Wx subunit groups from CS were distinguishable by different isoelectric points (pI). The HMW subunit of the Wx-A1 protein, known to be encoded by the *Wx-A1* gene on 7AS, was characterized by a more basic pI range than the other two. The LMW subunit group was composed of the *Wx-B1* and Wx-D1 proteins which had the same molecular weight but slightly different pIs. The Wx-D1 protein controlled by the *Wx-D1* gene on 7DS showed a more basic pI than the Wx-B1 protein produced by the *Wx-B1* gene on 4AL. The relative amounts of these two proteins were Wx-B1 > Wx-D1.

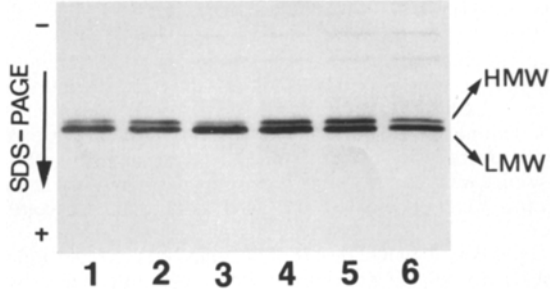
Compared to the CS euploid, the amount of Wx-B1 protein was reduced in monosomic 4A and a weakly-stained Wx-D1 protein was detected in monosomic 7D. As in Fig. 1, monosomic 7A had a very faint Wx-A1 protein. Thus, these results confirm the association of chromosomal locations of the three *Wx* genes with their corresponding Wx proteins. For monosomic 7B, the pattern was identical with that of CS. In the CNN monosomics for chromosomes 4A, 7A, 7B and 7D, a good agreement with the CS monosomics was detected for the banding patterns of the Wx proteins (data not shown), providing evidence for the occurrence of the *Wx-B1* gene on CNN 4A.

Table 2 The mean deviations for amylose content of the CS (CNN) substitution lines from the recipient parent CS, grown in the autumn-sown trials for three seasons, 1990–1992

Chromosome	Deviations from CS			Mean
	1990	1990/91	1991/92	
1A	−1.81**	0.40	0.08	−0.44
1B	−0.72	−0.35	0.13	−0.31
1D	−0.91	0.25	−0.40	−0.35
2A	−0.65	−0.33	0.05	−0.09
2D	1.02*	0.40	−0.35	0.35
3A	−0.14	−0.04	0.21	0.01
3B	−0.69	0.32	−0.26	−0.21
3D	−0.37	0.55	−0.01	0.06
4A	1.62*	0.85*	−0.01	0.82*
4B	0.16	0.48	0.32	0.32
4D	0.28	0.03	−0.01	0.10
5A	−0.83	0.26	0.13	−0.15
5B	−0.79	0.62	0.28	0.04
5D	0.12	0.43	−0.05	0.17
6A	−0.26	0.34	0.03	0.04
6B	−0.22	0.40	0.08	0.09
6D	−0.11	−0.18	−0.02	0.02
7A	−0.90	−0.08	0.40*	−0.20
7B	−1.89**	−1.21*	−0.67**	−1.25**
7D	0.19	0.11	0.08	0.13
CS	23.93	24.12	23.99	24.01
CNN	23.97	25.30	24.10	24.46

*,** Significantly different from CS at 5%, and 1% levels, respectively

Fig. 1 SDS-PAGE patterns of the Wx proteins from CS and monosomics for chromosome 4A and homoeologous group 7. The Wx proteins are detected as the high-molecular-weight (*HMW*) subunits and low-molecular-weight (*LMW*) subunits. *Lanes 1 and 6*, CS; *2*, monosomic 4A; *3*, monosomic 7A; *4*, monosomic 7B; *5*, monosomic 7D



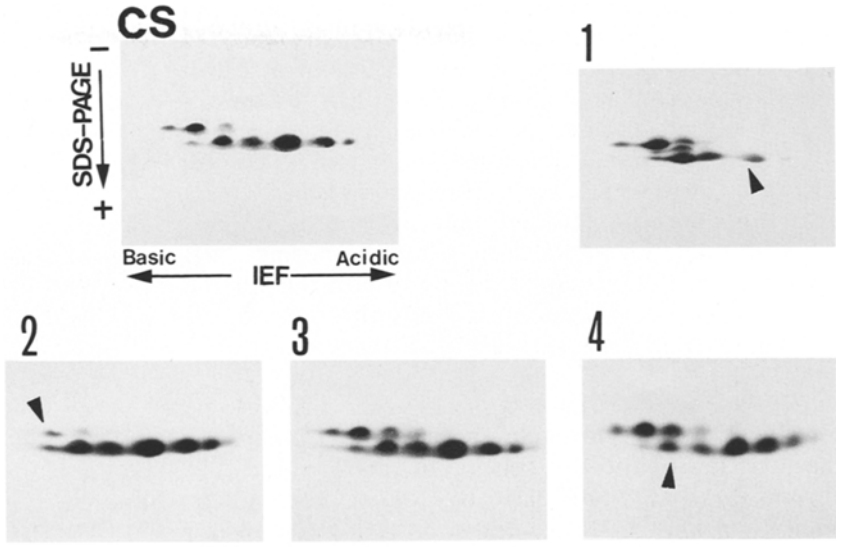


Fig. 2 2D-PAGE patterns of the Wx proteins from CS and the four monosomics. The reduced Wx proteins are *arrowed*. *1*, monosomic 4A; *2*, monosomic 7A; *3*, monosomic 7B; *4*, monosomic 7D

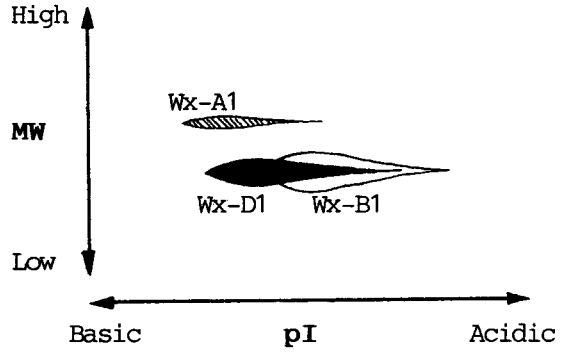


Fig. 3 A simplified diagram of Wx proteins from CS. Three distinct Wx proteins, Wx-A1, Wx-B1 and Wx-D1, are encoded by *Wx-A1* (7AS), *Wx-B1* (4AL) and *Wx-D1* (7DS) genes, respectively (Nakamura et al. 1993 a). The diagram shows differences in molecular weight (*MW*), isoelectric point (*pI*), and amount of the Wx proteins

Discussion

The endosperm starch of monosomic plants analyzed in this study was obtained from self-fertilized monosomics. As described by Payne et al. (1980), a majority or more than 70% of grains from a monosomic plant will have endosperms with only one dose of a critical chromosome compared with three for the disomic euploid. If amylose synthesis is related to gene dosage, it should therefore be possible to determine the chromosomal locations of the genes concerned. A similar model is expected for the electrophoretic analysis of the Wx proteins because monosomic grains lacking a critical chromosome are selected in advance and analyzed on a single-grain basis.

For amylose content, CS chromosomes 4A and 7B agreed with this gene-dosage model. Monosomic 4A produced lower-amylose-content grains than the euploid, indicating the location of a gene which either promotes the synthesis of amylose or suppresses the synthesis of amylopectin. The analysis of Wx proteins extracted from monosomic 4A and resolved by the 2D-PAGE system showed a reduction in the amount of the Wx-B1 protein, and again followed a gene-dosage effect. Thus it is plausible that the gene on CS chromosome 4A is responsible for the level of amylose content, and is identical to the *Wx-B1* gene which codes the Wx-B1 protein.

On the other hand, the short arms of CS chromosomes 7A and 7D are the sites of the *Wx-A1* and *Wx-D1* genes, respectively (Chao et al. 1989; Nakamura et al. 1993 a). Although the amounts of Wx proteins encoded by the *Wx-A1* and *Wx-D1* genes revealed gene-dosage effects (Figs. 1, 2), the amylose content in the monosomics for 7A and 7D showed no change from the CS euploid (Table 1). This contrasts with the behaviour of the Wx-B1 protein and its effect on amylose content. Nakamura et al. (1993 a) found from nullisomic analysis of CS that the relative amounts of protein were not the same for the three *Wx* genes; the amount of the Wx-B1 protein was the largest followed by the Wx-D1 and Wx-A1 proteins. This ranking was confirmed in the CS monosomics analyzed here. The simplest interpretation for the differential effects of the three *Wx* genes on amylose content is, therefore, in terms of the different potencies of these genes. Only a decrease in dosage of the most potent gene, *Wx-B1*, gives a sufficient reduction in the Wx-B1 protein to affect the level of amylose. A reduction in either of the Wx-A1 or Wx-D1 proteins can be compensated by the Wx-B1 protein.

Another important point arising from the present study is the significant effect of chromosome 7B on amylose content. An additional and separate gene from the *Wx* genes was suggested by the increased amylose content for CS monosomic 7B (Table 1) since CS chromosome 7B lacks the segment carrying the *Wx* locus

due to a reciprocal translocation with chromosome 4A (Naranjo et al. 1987; Liu et al. 1992). An involvement of chromosome 7B on amylose content was also shown from a significant reduction in the content for the CS (CNN 7B) substitution line (Table 2). These results suggest that CNN chromosome 7B, as well as CS 7B, carries an amylose gene apart from the *Wx* genes. This is also supported by the fact that while an increased amylose content occurred in CNN monosomic 7B (Miura et al. 1993) it could not be responsible for the action of the *Wx-B1* gene since CNN chromosome 7B lacks this gene as shown in 2D-PAGE analysis of the Wx proteins.

We have considered the possibility that chromosome 7B of wheat carries a regulator gene, which suppresses the activities of the *Wx* genes. On this model, CNN chromosome 7B would have to carry a more potent allele than CS 7B to account for the lower amylose content of the CS (CNN 7B) substitution line. This would also fit the behaviour of CNN monosomic 7B mentioned above. Furthermore, it might be of interest to consider the possibility that all the primitive group 7 chromosomes of wheat at one time carried homoeologous *Wx* genes as well as separate regulator genes, and that the translocation between 7B and 4A has separated the two genes. These possibilities are currently being investigated by mapping studies using the appropriate recombinant lines.

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