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Dosage effects of the three *Wx* genes on amylose synthesis in wheat endosperm

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Abstract Amylose synthesis in wheat endosperm is mainly controlled by the granule-bound starch synthase of about 60 kDa, the so-called waxy (Wx) protein. The Wx proteins are the product of the *Wx* genes at a triplicate set of single-copy homoeoloci located on chromosomes 7A (*Wx-A1*), 4A (*Wx-B1*) and 7D (*Wx-D1*). Using 'Chinese Spring' and its aneuploid lines, including nullisomic-tetrasomics, tetrasomics, ditelosomics and deletion stocks, together with single-chromosome substitution lines for these chromosomes, the effects of varying the dosage of whole chromosomes and chromosome arms, as well as the effects of null alleles, upon amylose synthesis were investigated. Nullisomic 4A and the deletion of chromosome segments carrying the *Wx-B1* gene reduced the amylose content by more than 3%. A reasonable agreement was found in the substitution lines. This confirms that the absence of the *Wx-B1* gene, or else substitution of this gene by its null allele, has the most striking effect on decreasing amylose synthesis. The removal of chromosomes carrying either the *Wx-A1* or the *Wx-D1* gene reduces the amylose content by less than 2%. A similar reduction was revealed by substitution of these two genes by the null alleles. Double dosages of chromosomes 7A, 4A and 7D did not increase amylose content, while the tetrasomic chromosomes produced more of the respective Wx proteins. This suggests that a certain level of *Wx* gene activity or of the Wx proteins led to the maximum amount of amylose.

Key words Amylose synthesis · Aneuploid · Granule-bound starch synthase · *Triticum aestivum* · *Wx* gene

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Introduction

The starch of normal cereal endosperm comprises two different forms of polymer, amylose and amylopectin. Amylose synthesis in the endosperm of diploid cereals such as maize and rice is controlled by the granule-bound starch synthase of about 60 kDa, the so-called waxy (Wx) protein (Tsai 1974; Echt and Schwartz 1981; Sano 1984). In hexaploid wheat (*Triticum aestivum* L.), evidence that the Wx protein is involved in amylose synthesis is provided by a close relationship between the amounts of the Wx protein and the amylose content (Yamamori et al. 1992; Miura and Tanii 1994). This protein is likely to play as important a role in endosperm texture and flour quality in wheat as it does in maize (Imam 1989) and rice (Sano et al. 1985).

Nakamura et al. (1993 a) have shown that the wheat Wx proteins can be resolved by two-dimensional polyacrylamide electrophoresis (2D-PAGE) into three subunit groups of polypeptides, and have used nullisomic-tetrasomic lines of the cultivar 'Chinese Spring' to attribute these groups to genes on chromosomes 7A, 4A and 7D respectively. The *Wx* genes, which are organized as a triplicate set of single-copy homoeoloci, are located on these three chromosomes (Chao et al. 1989; Miura et al. 1994). The amino-acid sequence predicted from a gene at one of these loci, probably *Wx-B1* on 4AL, is more than 83% identical to those of the *Wx* gene products of barley, maize and rice (Ainsworth et al. 1993).

Using monosomic lines, we have demonstrated that the three *Wx* genes, *Wx-A1* (7AS), *Wx-B1* (4AL which carries a translocation of a segment from 7BS) and *Wx-D1* (7DS), are not similar in their effects on changing the amylose content. Only a decrease in the dosage of the *Wx-B1* gene in the 4A monosomic gave a sufficient reduction in the Wx-B1 protein to reduce the level of amylose synthesis, whilst a reduction in either of the Wx-A1 protein in monosomic 7A or the Wx-D1 protein in monosomic 7D can be compensated for by the more abundant Wx-B1 protein (Miura et al. 1994). However, the influence of varying the dosage of the three *Wx* genes, from nullisomic to tetra-

somic level, on amylose content is not well documented. This paper assesses the effects of varying the dosage of whole chromosomes and chromosome arms carrying the *Wx* genes, as well as the effects of each null allele at the *Wx* locus, in an attempt to identify beneficial effects on quality that might be open to exploitation via cytogenetic manipulation or molecular biology.

Materials and methods

Genetic material

This study has focused on the spring cultivar 'Chinese Spring' (CS) and its available aneuploid lines and single-chromosome substitution lines to minimize effects due to genetic background. Two separate experiments were carried out employing the genotypes listed in Table 1.

In the first experiment, a three-season trial of nullisomic-tetrasomic (NT) and tetrasomic (T) lines, involving chromosomes 7A, 4A and 7D, were grown together with a CS euploid during 1993–1995 at the experimental field of Obihiro University, Obihiro, Japan. T7A was raised in only one season, 1993. T4A was complemented through the use of N4DT4A since their *Wx*-gene dosages in the endosperm are coincident (Table 1).

In the second experiment, the effects of a nullisomic dosage of the chromosome arms or segments on which the *Wx* genes are located were examined using the ditelosomic (DT) line for chromosome 7A and the deletion stocks for chromosome 4A by growing the materials both in the experimental field and in a glasshouse in the 1995 growing season. DT7AL is deficient for the short arm carrying the *Wx-A1* gene. Two deletion stocks for chromosome 4A of CS, developed by Endo and Gill (1995) and designated as 4AL-1 and 4AL-2, were examined instead of using DT4AS which has reduced fertility. These two stocks lack the segments of the long arm carrying the *Wx-B1* gene (Yamamori et al. 1994). No homozygous DT7DL line or deletion stocks for 7DS were available.

Table 1 Genotypes used and their *Wx*-gene dosage in the endosperm

Genotypes	*Wx*-gene dosage		
	Wx-A1	*Wx-B1*	*Wx-D1*
Nullisomic-tetrasomics (NT)			
N7AT7B	0	3	3
N7AT7D	0	3	6
N4AT4B	3	0	3
N4AT4D	3	0	3
N7DT7A	6	3	0
N7DT7B	3	3	0
Tetrasomics (T)			
T7A	6	3	3
N4DT4A[a]	3	6	3
T7D	3	3	6
CS euploid	3	3	3
Ditelosomic (DT)			
DT7AL	0	3	3
Deletion stocks			
4AL-1	3	0	3
4AL-2	3	0	3
Substitution lines			
CS(KT7A)	0	3	3
CS(KT4A)	3	0	3
CS(BH7D)	3	3	0
Donor parents			
Kanto107	0	0	3
Bai Huo	3	3	0

[a] T4A was complemented through the use of N4DT4A since their *Wx*-gene dosages are coincident

The effects of null alleles at each of the three *Wx* loci were also investigated using single-chromosome substitution lines for 7A, 4A and 7D. The Japanese spring wheat 'Kanto 107' was chosen as the donor parent for giving both null *Wx-A1* and null *Wx-B1* alleles, since it has been reported that this cultivar does not produce either the Wx-A1 or Wx-B1 proteins and only the *Wx-D1* locus is active (Nakamura et al. 1993 b). The null *Wx-D1* allele was derived from a Chinese spring wheat 'Bai Huo' which does not produce the Wx-D1 protein (Yamamori et al. 1994).

Using the conventional procedure described by Law and Worland (1973), the monotelosomics of CS as the recipient parent were pollinated by the disomics of the donor parents carrying null *Wx* alleles, followed by six backcrosses to the recipient parent and the extraction of disomic lines. Two lines for each substituted chromosome were developed as duplicates and thus the variation between these two lines could provide a measure of differences ascribable to genes other than those carried by the substituted chromosomes (Law and Worland 1973). One of duplicates for 'Bai Huo' 7D into CS was not included, due to an insufficient number of backcrosses at the time of this study. The substitution lines developed, and designated as CS(KT7A), CS(KT4A) and CS(BH7D), were checked for their validity by expression of the *Wx* gene products according to the electrophoretic methods described below. Furthermore, morphological marker genes, *Hd* on 4AS for the development of awns (Sears 1954) and *Rc3* on 7DS for red coleoptiles (Law 1966), were also readily identifiable.

The CS aneuploids were originally produced by Dr. E. R. Sears, University of Missouri, USA, and seed was provided from stocks maintained by Dr. Y. Furuta, Gifu University, Japan. The deletion stocks were kindly donated by Dr T. R. Endo, Kyoto University, Japan.

Growth conditions and measurement of amylose content

Each line planted in the field was represented by a single plot of 12 plants, spaced 10 cm between plants within a row and 30 cm between rows. Shortly after anthesis, the lines were covered to protect them from pre-harvest sprouting. Harvesting was carried out at maturity, and ears were threshed by hand. Grain samples were conditioned to about 15% moisture content and were milled on a Brabender Quadrant Junior Test Mill to produce a 60% extraction flour. Starch granules were separated from the flour using conventional methods. The amylose content per 100 mg of starch granules was colorimetrically determined as described by Miura et al. (1994). The assessment was carried out at least in triplicate. Student's *t*-tests were performed to detect significant differences between the CS euploid and each of the aneuploids or substitution lines.

SDS-PAGE of granule-bound proteins

For SDS-PAGE, starch granules were prepared as described by Echt and Schwartz (1981). An aliquot of 5 mg of dried starch granules was suspended in the extraction buffer (70 µl) and heated in boiling water for 5 min. The gelled starch solutions were cooled on ice and centrifuged at 15 000 rpm for 10 min. Using the supernatants, the expression of the Wx proteins was assessed by the 2D-PAGE system (Nakamura et al. 1993 a). Gels were 7.5% polyacrylamide with an acrylamide/bis-acrylamide ratio of 30:0.135 (w/w). Proteins were visualized by staining with silver-stain kits (Wako Pure Chemical Industry Ltd.).

Results

Amylose content

The variation in amylose content obtained from the first experiment of the NT and T lines is given in Table 2. The mean amylose content of the CS euploid across the three seasons was 25.52% (25.52 mg amylose per 100 mg starch granules) and its standard deviation between seasons was less than 0.5%. The effects associated with chromosome 4A were the most remarkable. N4AT4B and N4AT4D produced grains with about a 3%-lower amylose content than that of CS euploid, indicating that the absence of chromosome 4A, on which the *Wx-B1* gene is located, has a striking effect on the reduction in amylose content. A double dosage of this chromosome did not alter the amylose content since N4DT4A did not differ from CS in any season.

The influence of the removal of chromosomes 7A and 7D upon the changing amylose content was complicated by the combination of tetrasomic chromosomes. Significant reductions occurred in N7AT7B and N7DT7B, where the degree of reduction was about half compared to that of nullisomic 4A. On the other hand, no significant deviation from the CS euploid was detected in N7AT7D and N7DT7A. The amylose content of T7A and T7D was not different from that of CS. This revealed that, like chromosome 4A, double dosages of 7A and 7D had no effect on increasing amylose content.

The results for DT, deletion stocks, and substitution lines in the second experiment are presented in Table 3. The effects associated with chromosome 4A were again noticeable. The removal of chromosome segments carrying the *Wx-B1* genes in the two deletion stocks, 4AL-1 and 4AL-2, reduced amylose by 2%. Similarly, the substitution of CS chromosome 4A by its homologues from 'Kanto 107' brought about a practical reduction in amylose content. CS(KT4A) was about 2% lower than CS in the field-grown plants and 1% lower in the plants maintained in the glasshouse. The duplicate variation in this substitution line was slight, indicating that the number of backcrosses was sufficient to detect the character difference being studied.

Although the lack of chromosome arm 7AS, carrying the *Wx-A1* gene, did not bring about a practical reduction in amylose content, CS(KT7A) produced grains with about a 1% lower amylose content. This is attributable to an effect of 'Kanto 107' 7A carrying the null *Wx-A1* allele, rather than background effects, because differences between the two duplicate lines were negligible. For chromosome 7D, only one duplicate of CS(BH7D) in an environment was available. This substitution line produced grains with a 1% lower amylose content which was almost equivalent to that of CS(KT7A). Compared to the CS euploid, 'Kanto 107', the donor parent of chromosomes 7A and 4A, produced seeds with a 4 –5% lower amylose content.

Wx proteins

The *Wx* gene-dosage effects on the production of the Wx proteins were assessed with the 2D-PAGE system and the electrophotograms obtained are illustrated in Fig. 1. As in previous studies (Nakamura et al. 1993 a; Miura et al. 1994), the 60-kDa protein from the starch granules of the CS euploid could be resolved into two distinct subunit groups, the high-molecular-weight Wx-A1 protein and low-molecular-weight Wx protein. The latter further divided into two subunit groups of the Wx-B1 and Wx-D1 proteins with different isoelectric points.

Based on the band pattern of protein in the CS euploid, the lines could be separated into eight groups: three affecting the Wx-A1 protein, two affecting Wx-B1, and three affecting Wx-D1. Lack of the Wx-A1 protein was associated with the removal and substitution of chromosome 7A in N7AT7B, N7AT7D, DT7AL and CS(KT7A). Conversely, N7DT7A and T7A produced an increased amount of this protein. The Wx-B1 protein, which consists of more acidic

Table 2 The deviations for amylose content in the NT and T lines from the CS euploid, grown in the spring-sown trials for the three seasons, 1993–1995

Genotypes	1993	1994	1995	Mean
N7AT7B	–1.74*	–1.20*	–1.63***	–1.52**
N7AT7D	–0.56	0.22	0.29	–0.22
N4AT4B	–2.40***	–3.17***	–[a]	–2.78***
N4AT4D	–2.79***	–2.96***	–3.80***	–3.18***
N7DT7A	–0.68	0.32	–0.17	–0.18
N7DT7B	–1.80***	–1.48**	–0.72	–1.33**
T7A	0.13			
N4DT4A	–0.48	0.24	–0.09	–0.11
T7D	–0.11	–0.41	–0.80*	–0.44
CS euploid	25.94	25.39	25.23	25.52

*, **, ***; significant deviations from the CS euploid at the 5%, 1% and 0.1% levels, respectively
[a] Not included due to insufficient amount of starch

Table 3 The deviations for amylose content in the DT, deletion stocks, and substitution lines from the CS euploid, grown under the two environments in 1995

Genotypes	Field	Glasshouse	Mean
DT7AL	–0.20	–0.15	–0.18
CS(KT7A)-1[a]	–0.72	–1.23*	–0.95*
CS(KT7A)-2	–1.07*	–1.05*	–1.06*
4AL-1	–1.56**	–2.29***	–1.92***
4AL-2	–2.23***	–2.52***	–2.38***
CS(KT4A)-1	–2.14**	–1.24**	–1.69***
CS(KT4A)-2	–2.61**	–1.05**	–1.83***
CS(BH7D)-1	–1.03**		
Kanto 107	–4.31***	–5.43***	–4.87***
Bai Huo	–0.67*		
CS euploid	25.23	25.10	25.16

*, **, ***; significant deviations from the CS euploid at the 5%, 1% and 0.1% levels, respectively
[a] Duplication lines in each substitution line, see text

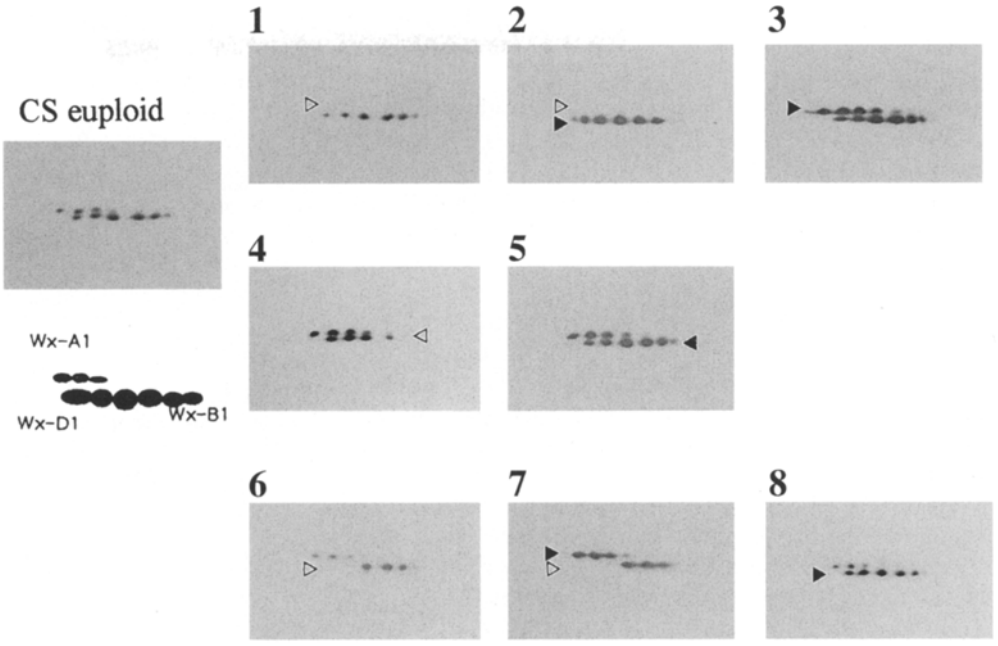

Fig. 1 **1–8** 2D-PAGE patterns of the Wx proteins from the CS euploid and eight groups of aneuploid lines and substitution lines. Group **1** pattern is in N7AT7B, DT7AL, CS(KT7A) : **2** in N7AT7D : **3** in T7A : **4** in N4AT4B, N4AT4D, 4AL-1, -2, CS(KT4A), **5** in N4DT4A : **6** in N7DT7B, CS(BH7D), **7** in N7DT7A, **8** in T7D. *Empty arrows* indicate the absence of a particular Wx protein. *Closed arrows* indicate an increased amount of the Wx protein

subunits than the Wx-D1 protein, was either lacking or greatly reduced in N4AT4B, N4AT4D, DN4AL-1, -2 and CS(KT4A). For the Wx-D1 protein, the removal or substitution of chromosome CS7D did not produce this protein as in N7DT7A, N7DT7B and CS(BH7D). N4DT4A produced more of the Wx-B1 protein, like N7AT7D and T7D with an increased amount of the Wx-D1 protein.

These results indicate that an increase in the gene dosage effect due to the presence of tetrasomic chromosomes is reflected in an enhanced amount of protein. However, whether the amount of the respective Wx proteins was proportionally twice in tetrasomics was not defined.

No duplicate differences in each set of the substitution lines were seen in the 2D-PAGE analysis (data not shown).

Discussion

A close association of a reduction in amylose content with lack of the Wx-B1 protein was detected in the nullisomic 4A and the two deletion stocks, as well as in CS(KT4A). This has demonstrated that the gene on CS chromosome 4A is responsible for the level of amylose synthesis and is probably identical to the *Wx-B1* gene because it was found that the deletion stocks lacked the very small region on which the *Wx-B1* gene is located (Yamamori et al. 1994). The analysis of the Wx proteins extracted from N7AT7B and N7DT7B and resolved by the 2D-PAGE system have also revealed that lack of the Wx-A1 and Wx-D1 proteins, respectively, is connected with a reduction in the amylose content of these lines. The fact that lack of the Wx-A1 protein in CS(KT7A) or of the Wx-D1 protein in CS(BH7D) can decrease the amylose content confirms the above results. Hence the reduction in amylose content by the removal of whole chromosomes or chromosome segments, as well as the substitution of CS7A, 4A and 7D, can be ascribed to absent or silent *Wx* genes. This conclusion is supported by the fact that waxy wheat which carries null alleles at all three *Wx* loci is deficient in all of the three Wx proteins and does not produce amylose (Yamamori et al. 1995). Only the behavior of DT7AL, with a lack of the Wx-A1 protein but almost no reduction in amylose content, could not be explained by this *Wx* gene-dosage effect. Therefore, the possibility that confounding effects from other loci in the same linkage block on chromosome 7A are involved in amylose synthesis should be examined further.

The reduction in amylose content by the removal of chromosomes 7A and 7D in N7AT7B and N7DT7B, respectively, suggests that, even if the *Wx-B1* gene is active, the complete lack of either the *Wx-A1* or *Wx-D1* genes, as well as substitution by their null alleles, can reduce amylose content to a certain degree. It is of interest to know which of *Wx-A1* or *Wx-D1* is more potent in its effect on amylose synthesis. To answer this question, under the assumption that effects associated with chromosome dosage are exclusively due to the *Wx* genes, a comparison between N7AT7D and N7DT7A, as well as that between CS(KT7A) and CS(BH7D), would provide a solution. No deviation in amylose content from the CS euploid was detected in the two NT lines, which carry the same number of *Wx* genes as CS, by removal of *Wx-A1* and the addition of *Wx-D1* in N7AT7D and vice versa in N7DT7A. Therefore, the effects of a nullisomic chromosome seem to be offset by the effect of the tetrasomic chromosome, suggesting that there is no distinctive difference in amylose synthesis between these two genes. This is supported by evidence that

CS(KT7A) and CS(BH7D) have a very similar level of amylose content with about a 1% reduction from the CS euploid.

The effects of gene dosage on amylose content were not the same among the three *Wx* genes. The dosage effect of the *Wx-B1* gene was found to be linear from a zero dose in the endosperm of nullisomic 4A to three doses in that of the euploid. Compared to the CS euploid about a 3% reduction in the nullisomic 4A was followed by a 2% reduction in monosomic 4A having one dose of the gene (Miura et al. 1994), and then a plateau arose between 3–6 doses in the tetrasomic 4A (N4DT4A in this study). On the other hand, for the *Wx-A1* and *Wx-D1* genes, a reduction in amylose content occurred only at a zero dose in each of the nullisomics 7A and 7D but it disappeared when at least one dose is active. One possible explanation for this is due to compensation by the most abundant Wx-B1 protein. These findings complement our previous conclusion using monosomic lines that the action of the *Wx-B1* gene is predominant in amylose synthesis (Miura et al. 1994).

Another important point arising from the present study is the behavior of tetrasomic chromosomes. All of the three tetrasomic chromosomes carrying the double dosage of the *Wx* gene produced an increased amount of the respective Wx proteins in the NT and T lines (Fig. 1). However, amylose synthesis was not enhanced by the increased Wx protein, indicating that none of the three *Wx* genes could alter the amylose content at the tetrasomic level or at the level of the six doses present in the endosperm. This suggests that a certain level of the *Wx* gene activity, or else of the Wx protein, led to a maximum amount of amylose, and further increases in the *Wx* gene products do not bring about any additional increase in amylose content. A similar finding, that the amount of the Wx protein is not linearly proportional to the amylose content in the endosperm, has been revealed in maize (Tsai 1974; Echt and Schwartz 1981), rice (Sano et al. 1985) and potato (Flipse et al. 1996).

As mentioned earlier, the complete lack of the *Wx-B1* gene, or else substitution by its null allele derived from 'Kanto 107' 4A, are the most potent in reducing amylose content. This combination of inactivity of null *Wx-B1* with a low amylose content may explain the fact that there is a high frequency of null *Wx-B1* alleles in Australian and Japanese cultivars accepted into noodle classification in which the low amylose content is a common property (Miura and Tanii 1994; Yamamori et al. 1994; Zhao and Sharp 1995). A large decrease in amylose content of 'Kanto 107' is caused by the null *Wx-A1* and null *Wx-B1* genes (Nakamura et al. 1993 b). Furthermore, a small but significant decrease in amylose content was observed in CS(KT7A) and CS(BH7D) showing that the null *Wx-A1* and null *Wx-D1* alleles contribute to reducing amylose synthesis to some extent. In order to breed wheats with diverse starch composition, this should prompt us to survey and develop the other two types of low-amylose-content wheats, one of which carries the null *Wx-A1* and null *Wx-D1* alleles and the other the null *Wx-B1* and null *Wx-D1* alleles. The production of such wheats has not yet been achieved (Yamamori et al. 1994). Since the development of wheats with two sets of null *Wx* genes using our three substitution lines, CS(KT7A), CS(KT4A) and CS(BH7D), should be of considerable interest, this project is now in progress.

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