コムギの*Wx*遺伝子と量的遺伝子座の連鎖分析

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はしがき

ここ 3 年にわたって、カナダのサスカチュワン州、モンゴル高原、中国西域の小麦栽培地帯を訪れる機会に恵まれた。いずれも乾燥した真夏の陽光が照りつける時期で、黄金色に稔った小麦たちは収穫間近であった。地平線まで続くカナダの小麦畑、遊牧地とのコントラストが美しいモンゴル小麦、一方集約栽培の中国西域シルクロード沿線では、太陽の恵みを一粒たりとも粗末にしない農民の姿が印象的であった。

太陽エネルギーを利用して作り出したスクロースを種子胚乳中で貯蔵デンプンに変換する一見単純そうな機構も、デンプン生合成に関わる遺伝子側からみたときその理解や育種的改変は必ずしも容易でない。本研究はそれら一連の変換過程のごく一部、具体的にはデンプン中のアミロース合成に関わる *Wx* 遺伝子を対象に解析したものである。得られた知見が将来の小麦生産に少しでも役立てばと願いながら、中緯度地帯の澄み切った大空の下で眩かった小麦を思い出している。

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研究発表

(1) 学会誌等

Miura H., Wickramasinghe M.H.A., Subasinghe R.M., Araki E. and Komae K. : Development of near-isogenic lines carrying different null *Wx* alleles of wheat and their starch property. Euphytica (submitted to)

Araki E., Miura H. and Sawada S. : Differential effects of the null alleles at the three *Wx* loci on starch-pasting properties in wheat. Theoretical and Applied Genetics 100: 1113—1120, 2000

Miura H., Araki E. and Tarui S. : Amylose synthesis capacity of the three *Wx* genes of wheat cv. Chinese Spring. Euphytica 108: 91—95, 1999

Araki E., Miura H. and Sawada S. : Identification of loci affecting amylose content and agronomic traits on chromosome 4A of wheat. Theoretical and Applied Genetics 98: 977—984, 1999

Araki E., Miura H., Watanabe N. and Sawada S. : Variation for amylose content in wheat cultivars carrying different null alleles at the *Wx* loci. Research Bulletin of Obihiro University 21: 9—15, 1998

Araki E., Miura H., and Sawada S. : Genetic analysis of the amylose synthesis gene *Wx-B1* and QTL affecting agronomic traits on chromosome 4A of wheat. *In*: Slinkard A.E. (ed.), Proceedings of the 9th International Wheat Genetics Symposium, Saskatoon, Canada, Vol. 2: 144—146, 1998

Miura H., Tarui S., Araki E. and Nakagawa Y. : Production of Wx-protein deficient lines in wheat cv. Chinese Spring. *In*: Slinkard A.E. (ed.), Proceedings of the 9th International Wheat Genetics Symposium, Saskatoon, Canada, Vol. 4: 208—210, 1998

Watanabe N. and Miura H. : Diversity of amylose content in *Triticum durum* and *Aegilops tauschii*. *In*: Slinkard A.E. (ed.), Proceedings of the 9th International Wheat Genetics Symposium, Saskatoon, Canada, Vol. 4: 291—293, 1998

(2) 口頭発表

荒木悦子・三浦秀穂・沢田壮兵：デンプン特性に関わるコムギ 4A 染色体短腕上の遺伝子座. 日本育種学会第 94 回講演会　岩手大学農学部　1998 年 9 月

荒木悦子・江熊香織・三浦秀穂・沢田壮兵：コムギデンプンの糊化特性に対する *Wx* 座の影響. 日本育種学会第 95 回講演会　宇都宮大学農学部　1999 年 4 月

Subasinghe Renuka・荒木悦子・谷口悦子・小前幸三・三浦秀穂：コムギ Wx タンパク質欠失系統のデンプン特性の変異. 日本育種学会第 97 回講演会　筑波大学　2000 年 4 月

三浦秀穂・長　学・柳沢　朗・菅原章人：コムギ Wx タンパク質欠失系統における小麦粉特性の地域と年次変動. 日本育種学会第 98 回講演会　弘前大学農学部　2000 年 9 月

(3) 談話会報

Subasinghe R.・三浦秀穂：Developing wheat doubled haploid lines carrying the different null *Wx* alleles. 日本育種学会・作物学会北海道談話会報 39 : 81-82, 1998

三浦秀穂・江熊香織・荒木悦子：コムギ胚乳のアミロース含量とタンパク含量の遺

伝的関係. 日本育種学会・作物学会北海道談話会報 39 : 83-84, 1998

Wickramasinghe, M.H.A.・千葉健太郎・三浦秀穂・加藤清明・沢田壮兵 : Gene-dosage effects of individual *Wx* loci on amylose content in wheat. 日本育種学会・作物学会北海道談話会報 41(印刷中), 2000

Linkage analysis of the wheat *Wx* genes and quantitative trait loci

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Chapter 1. Backgrounds of the present research

1.1 Cereal starch as human diet

Crop plant domestication represents one of the mankind's greatest achievements. Cereals are the main components of human diet and are crucial to human survival. More than 40 percent of the food consumed by man is derived from the grains of two cereals, wheat (*Triticum aestivum* L. em. Thell.) and rice (*Oriza sativa* L.). Nearly 35 percent of the world's population uses wheat starch as the major source of calories in diet, while rice is the staple food of over half the world's people (Blakeney, 1996).

The functional and nutritional quality of cereal grains determines their suitability for specific purposes and may limit the quality of the end-use product, influencing greatly the commercial value of grain. An understanding of the factors that control grain quality is thus important in the maintenance of efficient and sustainable agriculture and food production. End-uses of the cereal grains are dependent on several determinants including starch composition and quality, protein quantity and quality, content of lipid and lipoprotein, and pigments of endosperm and pericarp (Morris, 1998). Usually cereal grains consist of the embryo, endosperm and pericarp, especially the endosperm is major part of the grain and accounts for more than 80% of the grain weight. The predominant reserve carbohydrate found in cereal endosperm is starch which comprises approximately three-fourths of the endosperm and plays an important role in determining the end-uses of cereal grains. Hence, a knowledge of biochemical properties of the endosperm starch and its genetics will be of great value for plant breeding purposes and for manipulating genes of agronomic and economic interest.

1.2 Morphology and structure of starch granule

The starch formed in all organs of plants is packaged into starch granules, which vary widely between species and organs in size and shape. Major attention has been devoted to the storage starch which accumulates as a complex granular structure in the amyloplast of endosperm. Sizes vary from 1μm up to 100 μm. In mature wheat, barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.) and oat (*Avena sativa* L.) grains, a bimodal distribution of starch granule size is observed, being composed of a lenticular shaped 'A' granule population of 10-30 μm in diameter, and a spherical 'B' granule population with a diameter less than 10 μm (Stark and Lynn, 1992). While the protein complement is identical between the granules (Rahman et al., 1995), how and why these different populations are

deposited in the plant tissue is not yet explained with satisfaction. Also effects of the 'A' and 'B' granules on starch quality are not well defined.

The endosperm starch consists of two structurally different forms of polymers, amylose and amylopectin (Fig. 1). Amylose is composed of linear chain component of α-(1-4) linked D-glucose residues, but some molecules are slightly branched by (1-6)-α-likages. On the other hand, amylopectin is the highly branched chain component where the chains of α-(1-4) linked D-glucose branch every 20-25 residues through the (1-6)-α-likages (Shannon and Garwood, 1984).

For the granule structure, Smith et al. (1997) and Buleon et al. (1998) reviewed that the chains within the starch granule are radially arranged with their nonreducing ends pointing toward the surface, and organized into alternating crystalline and amorphous lamellae. The major crystalline amylopectin fraction is radially oriented in the granule with the characteristic ring structures being formed by alternating clusters of herices formed by external amylopectin chains. So amylopectin is usually assumed to support the framework of the crystalline regions. The nature and size of growth rings within the granule is highly dependent on growth conditions. While amylose content increases during starch granule development (Morrison and Gadan, 1987), very few reports are available concerning the conformation of amorphous amylose and amylopectin. Similarly, the respective contribution of amylose and amylopectin to crystallinity, the size distribution of crystalline areas or the organization of mixed 'A' and 'B' granules remain unresolved.

1.3 Amylose content and starch quality

As mentioned above, the starch granule organization is very complicated and there are many unsolved problems. Nevertheless, there is general agreement that the amylose/amylopectin composition is an important criterion in determining the end-uses of plant starch in food and non-food industries. In most common types of cereal endosperm starch, the relative composition of amylose and amylopectin ranges between 72 and 82% amylopectin, and 18 and 33% amylose (reviewed by Buleon et al., 1998). In addition, some mutant genotypes of maize (*Zea mays* L.), barley, and rice contain as much as 70% amylose, whereas other genotypes, called waxy, contain less than 1% amylose or amylose-free. In dicot crop species, the amylose-free potato (*Solanum tuberrosum* L.) and low amylose mutants of pea (*Pisum sativum* L.) are also developed and noticed (Hovenkamp-Hermelink et al., 1987; Denyer et al., 1995a).

When plant starch is used industrially either as a thickening agent, flavor carrier or binder in food systems, its effectiveness depends upon the ratio of amylose to amylopectin (hereafter referred to as 'amylose content'), as well as their organization within starch granule (Zobel, 1984). Starch

with high amylose content is also useful in non-food industry, because plant starch may improve the texture and quality of plastics to be almost completely degraded within a short time (Watanabe et al., 1998). On the other hand, the amylose-free starch of maize and potato has been noted in the both food and non-food industries for its paste clarity, high water binding capacity, resistance to gel formation and retrogradation (Visser and Jacobsen, 1993; Zeng et al., 1997; Graybosch, 1998). The waxy or glutinous rice is traditionally valuable in the production of certain foods such as rice cakes and porridge (McKenzie and Rutger, 1983; Sano et al., 1986). Recently, a waxy wheat has been produced by classical breeding (Nakamura et al., 1995) and is a current topic of discussion as the processing properties of waxy wheat are undoubtedly different in many respects to normal or wild-type genotypes (Hayakawa et al., 1997; Fujita et al., 1998).

1.4 Biochemical pathway of starch synthesis

To improve starch quality by changing starch composition, a better understanding of the factors controlling amylose and amylopectin synthesis is required. Intricate details for the biochemical pathways of starch synthesis have been reviewed by many researchers (Keeling et al., 1988; Tetlow et al., 1994; Morell et al., 1995; Martin and Smith, 1995; Ball et al., 1996; Wang et al., 1998; Buleon et al., 1998; Smith, 1999).

The pathway leading to synthesis of starch consists of four enzymatic reactions (Fig. 2), ADP-glucose pyrophosphorylase (ADPase), starch synthase (SS), starch branching enzyme (SBE) and starch debranching enzyme (SDE). First hexose phosphates such as glucose-1-phosphate, glucose-6-phosphate, or fructose-6-phosphate enter into the amyloplast from cytosol. The ADPase is responsible for the synthesis of ADP-glucose, the substrate for starch synthesis. Then ADP-glucose is converted to α-(1-4) linked D-glucose residues to form amylose by SS. ADP-glucose is also converted to α-(1-6) linked D-glucose residues to form amylopectin by the interaction between SS and SBE. Although amylose and amylopectin are synthesized in two independent pathways, it is found that these two polymers can inter-convert due to actions of the enzymes such as SBE and SDE which digest selectly α-(1-6) linked D-glucopyronosyl.

1.4.1 Starch synthase (SS)

SS catalyses the ADP-glucose conversion into amylose and amylopectin by the two independent pathways (Fig. 2). On the basis of solubility properties of enzymes when extracted from starch granules, SS is divided into two classes, soluble starch synthases (SSS) which can separate partly from the starch granules, and granule-bound starch synthases (GBSS) which are exclusively

granule-bound. It is widely accepted that SS in the soluble fraction of the amyloplast elaborates amylopectin at the surface of the starch granule. On the other hand, SS tightly bound to starch granules synthesizes amylose within the starch granule.

1.4.2 Soluble starch synthase (SSS)

Most starch synthesizing organs contain multiple isoforms of SSS responsible for amylopectin synthesis. These isoforms can be divided on the basis of their amino-acid sequences into three distinct classes, named SSS I, SSS II and SSS III. All the three classes have been identified in potato tuber (Edwards et al., 1995) and maize endosperm (Mu et al., 1994). In other crop plants, some isoforms of SSS have been purified from rice endosperm (55 and 57 kDa, Baba et al., 1993) and pea embryo (77 and 60 kDa, Denyer and Smith, 1992). All plant starches probably contain at least one isoform of SSS (Smith et al., 1995). In wheat, the 75 kDa protein was found to have the SSS activity which was antigenically related to the pea 77kDa SSS (Denyer et al., 1995b).

To date, a number of genes for SSSs have been identified and characterized, which include SSS I in rice (Baba et al., 1993), SSS I, II, and III in potato (Edwards et al., 1995), SSS Ia and SSS Ib in maize (Mu et al., 1994), SSS I and SSS II in pea (Dry et al., 1992). The wheat SSS cDNA sequences exhibited 55-70% similarity to maize SSS Ia and SSS Ib, and thus were considered to be counterparts of the maize SSS (Gao and Chibbar, 1998).

It is recognized that the enzymatic activity of SSS can be reduced either by mutation in the coding genes, e.g., in barley and pea (Tynela and Schulman, 1993), or by an increase in growth temperature during endosperm development of wheat (Jenner, 1994). However, the precise effect of reduction in the SSS activity on starch synthesis is unknown. Recent studies using transgenic potatoes (Edwards et al., 1995) have shown that specific inhibition of the gene encoding the 70 kDa SSS I, did not change any structural properties of starch, whereas the repression of the SSS II gene, encoding the 85 kDa protein, led only slight changes in the starch structure. However, repression of the genes encoding the 135kDa SSS III, reduced the amylose and phosphate contents and altered the gelation characteristics. These observations indicate that the individual SSS isoforms might have different roles in starch synthesis, but details are not clear for the most part. Further, Edwards et al. (1999) reported that synergistic manner between SSS II and SSS III for amylopectin structure and starch viscosity in potato tubers.

1.4.1.2 Granule-bound starch synthase (GBSS)

Much of the granule-bound protein activity in storage organs is attributable to a class of isoform

known as granule-bound starch synthase I (GBSS I), with a molecular weight of 59-60 kDa (Preiss and Levi, 1980). This enzyme has been called as the waxy (Wx) protein in cereals, because the waxy or amylose-free phenotype is originally characterized in maize by the significant reduction of the GBSS I activity and by loss of the 59-60 kDa protein (Tsai, 1974; Nelson et al., 1978; Echt and Schwarz, 1981). The observation that waxy mutants from barley, maize, rice, pea and potato lack the GBSS I isoform demonstrates in turn that this enzyme activity is responsible solely for the amylose synthesis (Martin and Smith, 1993).

The genes affected by these waxy mutations are referred to as the *waxy* (*Wx*) genes in cereals, *amylose-free* (*amf*) in potato and *low amylose* (*lam*) in pea. The dosage effects of the genes for GBSS I on the enzyme activity and amylose content in the triploid endosperm tissues were first analyzed in maize (Tsai, 1974), subsequently in rice (Sano et al., 1986). The dosage effect of the *amf* allele in potato tuber is also investigated (Flipse et al., 1996a). Those studies verified that the dosage of the mutant null alleles is linearly and negatively correlated to the GBSS I activity but not to amylose content.

The genes encoding GBSS I have been cloned and characterized in various plants, such as maize, rice, potato, pea and barley, and then its information is applied for analysis. The introduction and expression the antisense RNA of the genes for GBSS I inhibited the activity of the enzyme and significantly reduced amylose content in rice endosperm (Shimada et al., 1993) and potato tuber (Visser et al. 1991).

1.4.2 Starch branching enzyme (SBE)

The nature of SBE that catalyzes the formation of the α-1,6 linkages of amylopectin might be expected to be of great importance in determining amylopectin synthesis. The fact that plant organs almost invariably contain multiple isoforms of SBE (Burton et al., 1995; Martin and Smith, 1995) raises a possibility that multiple forms of SBE could give raise to variation in branching pattern and polymodal distribution of chain lengths which underlie the cluster structure of amylopectin. There are two classes of isoforms in plants, SBE I (or class B) and II (or class A) with about 85-90 kDa in mass. The first class includes maize SBE I, rice SBE I, pea SBE II and potato SBE, while the second contains maize SBE II, pea SBE I and rice SBE III. The discrepancy among plants is partly due to the fact that the nomenclature was based on their order of elution from maize endosperm during extraction of enzymes (Morell et al., 1995).

The genes or cDNA for SBE have been isolated from maize SBE I (Baba et al., 1991) and II (Fisher et al., 1993), rice SBE I (Kawasaki et al., 1993) and III (Mizuno et al., 1993), pea SBE I and

II (Smith, 1988; Burton et al., 1995) and potato SBE (Poulsen and Keiberg, 1993).

The roles of SBE have been investigated through study of mutant and transgenic plants. Smith et al. (1997) reviewed that mutations at the *amylose-extender* (*ae*) loci of cereals and the *rugosus* (*R*) locus of pea lead specifically to the loss of an A isoform, but no dramatic change in the distribution of chain lengths occurs. In transgenic potato produced by introduction of the antisense RNA of the SBE gene, the repression of the native SBE gene altered the structure of some soluble glucans but did not result in any increase of amylose content and had only minor effects on the structure of amylopectin (Flipse et al., 1996b). The SBE I and II isoforms in maize endosperm differ both in their substrate affinities and in the length of branches they preferentially create. In vitro, SBE II preferentially branches amylopectin, whereas SBE I preferentially branches amylose. In vivo, however, these two isoforms do not seem to play distinct and essential roles in creating the cluster structure of amylopectin (e.g. Smith et al., 1997).

1.4.3 Starch debranching enzyme (SDE)

The available evidence suggests that SDE plays a role in the determination of structure and composition of amylopectin, and thus the end-uses of starch (Ball et al., 1996; Repellin et al., 1998). The enzymatic properties of SDE have been described (Pan and Nelson, 1984; Doehlert and Knutson, 1991; Smith et al., 1997). Multiple isoforms have been identified and two types of enzymes, isoamylase in maize and limit dextrinase (or pulanase or R-enzyme) in rice have been found to have the SDE activity. However, compared to SS and SBE, SDE is the least well characterized of the starch biosynthetic enzymes, and information on the biochemical properties and roles in starch synthesis is rather limited. There are no reports that SDE is bound within the starch granules during the endosperm development.

1.6 The genes controlling starch synthases in wheat

Starch granules isolated from the developing and mature endosperm of wheat contain two classes of proteins that possibly involve in starch synthesis; proteins which are embedded within the granule and loosely associated surface proteins (Schofield and Greenwell, 1987; Rahman et al., 1995). As summarized in Table 1, it is possible by gel electrophoresis on the basis of size to resolve the granule-bound proteins into four starch syntheses, GBSS I, SGP-3, SGP-2 and SGP-1 with molecular weights of 60, 75, 85, 100 - 115 kDa, respectively (Denyer et al., 1995; Rahman et al., 1995; Yamamori and Endo, 1996; Takaoka et al., 1997), while the precise molecular weights of these proteins vary slightly depending on the details of the gel electrophoresis system employed.

The genes coding these enzymes have been identified and sequenced using a range of molecular technology.

1.5.1 *Wx* genes

The most prominent GBSS I or Wx protein is responsible for amylose synthesis and is encoded by the *Wx* locus. Chao et al. (1989) demonstrated by the RFLP analysis of the group-7 chromosomes using a cDNA clone from the barley Wx protein (pcwx27; Rohde et al., 1988) as the DNA probe that each wheat genome possesses one homoeologous *Wx* locus, *Wx-A1* on the short arm of 7A, *Wx-B1* on the long arm of 4A and *Wx-D1* on the short arm of 7D. This was confirmed by means of Southern blots analyses (Clark et al., 1991; Ainsworth et al., 1993a) and by the electrophoretic analysis of the Wx proteins (Nakamura et al., 1993a). The fact that *Wx-B1* was found on 4AL not on 7BS result from that the segment of the short arm of the original 7B containing the *Wx* locus has been translocated to the long arm of 4A (Nalanjo et al., 1987; Devos et al., 1995).

Ainsworth and coworkers first cloned and sequenced a wheat *Wx* cDNA (Clark et al., 1991; Ainsworth et al., 1993a). Analysis of the sequence of 615 amino acids and alignment with five other plant Wx proteins (barley, maize, rice, potato and pea) showed that they exhibit substantial homology (Ainsworth et al., 1993a). But it was not clarified which wheat *Wx* gene of the three genome. Recently Murai et al. (1999) have reported complete genomic DNA sequences of the three homoeologous *Wx* structural genes. Each *Wx* gene consists of 11 exons and ten introns, with variation in length from start to stop codon, 2781bp in *Wx-A1a*, 2794 bp in *Wx-B1a* and 2862 bp in *Wx-D1a*. But they are closely similar to one another in the nucleotide sequences, with 95.6-96.3% homology in mature protein regions, while only *Wx-B1a* contains a trinucleotide insertion (CAA) in the region encoding the transit peptide.

On the other hand, Vrinten et al. (1999) characterized the null mutations at the three wheat *Wx* genes. The null *Wx-A1b* and *Wx-D1b* alleles contain small DNA insertions, or filler DNA. As a result, the functional Wx-A1 and Wx-D1 proteins are not produced, although the transcriptions occur. The null *Wx-B1b* allele has sustained a deletion which includes the entire coding region of the *Wx-B1* gene.

A different wheat GBSS isoform, designated GBSS II, was detected in the pericarp starch granule but not endosperm (Nakamura et al., 1998). The GBSS II is not identical to the wheat GBSS I in amino acid sequence but shows strong homology to GBSS Is of other cereals and potato, and contains the motif KTGGL, which is deduced as the substrate-binding site of GBSS I.

1.5.2 Genes encoding the starch granule proteins

The 75 kDa protein (Rahman et al., 1995) or SGP-3 (Yamamori and Endo, 1996) found both in soluble and granule-bound fraction, and peptide sequencing showed to be structurally similar to rice SSS (Takaoka et al., 1997). Yamamori and Endo (1996) and Li et al. (1999) provided evidence that the 75 kDa protein consists of three isoforms, each encoded by the homoeologous genes flanked with centromere and the *Wx* locus on the short arms of group 7 chromosomes.

The 85 kDa protein (Rahman et al., 1995) or SGP-2 (Yamamori and Endo, 1996) has SBE activity. The cDNA (Repellin et al., 1997) and genomic DNA (Rahman et al., 1997) for the wheat SBE I as well as cDNA for the SBE II (Nair et al., 1997), have been cloned recently. The three isoforms of the wheat SBE I are encoded by the genes located on the homoeologous group-7 chromosomes (Morell et al., 1997; Rahman et al., 1999), whereas the wheat SBE II is possibly the products of genes on the group-2 chromosomes (Sharp, 1997).

Unlike the 75 and 85 kDa proteins, a group of three proteins of 100 - 115 kDa or the SGP-1 proteins is suggested to be wheat specific since surveys of other cereals indicate that analogous proteins to the SGP-1 are not present (Rahman et al., 1995; Yamamori and Endo, 1996). The 100-, 108-, and 115 kDa proteins designated as SGP-B1, SGP-D1 and SGP-A1 have been shown to be encoded at homoeologous loci on the short arms of chromosomes 7B, 7D and 7A, respectively (Denyer et al., 1995; Yamamori and Endo, 1996; Yamamori et al., 2000). Li et al. (1999) reported the cloning of cDNAs for each of the SGP-1 polypeptides and demonstrated that they are member of the SS II. It may be of interesting to notice that the co-location on the group-7 chromosomes of the genes for enzymes related to starch synthesis is striking (Table 1).

1.6 Objectives of the present study

The advent of routine genetic manipulation of the enzymes catalyzing starch synthesis in the wheat endosperm has made it possible, in theory at least, to change the physical and chemical characteristics of starch and to provide novel variation in raw materials. This concept has led to a better understanding and improvement of grain protein quality and quantity (e.g. Wrigley and Morris, 1996). However, it has only been in the last decade so that wheat scientist and breeder have recognized that genetic variation for starch properties, starch composition in particular, is responsible for the variation in end-use quality. Therefore an improved understanding of the genes coding amylose and amylopectin synthesizing enzymes is needed, as well as linkage relationships between 'starch genes' and agronomic trait loci.

The present study was carried out to define and discuss the contributions of the homoeologous

three *Wx* genes of wheat to variation for amylose content, starch structure, starch pasting properties and important agronomic traits. The subjects focused in this study are as follows. Amylose synthesis capacity of the three *Wx* genes was determined in Chapter 2 using eight possible types with different combinations of wild type and null alleles at the *Wx* loci. Then the starch molecular structures and field performance in the near-isogenic eight types were compared in Chapter 3. The contributions of *Wx-B1* on chromosome 4A to agronomic traits, as well as QTL for amylose content were analyzed in Chapter 4. In Chapter 5, the effects of the null alleles at the *Wx* loci on starch-pasting properties were distinguished using three separate sets of RSLs for chromosomes 7A, 4A and 7D.

1.7 Gene symbols used

The gene symbols used in this thesis were conformed to "Recommended Rules for Gene Symbolization in Wheat" (McIntosh et al., 1998), which was adapted from the International Rules of Genetic Nomenclature.

The nomenclature recommended is that the basic symbol for a gene locus should consist of a two-, three-, or four-letter abbreviation of the trivial name of the enzyme or protein and the initial letter should be a capital. The genes encoding the wheat GBSS I have been designated variously, such as *WAXY*, *waxy*, *Wx*, *wx*, *GBSS-7A*, or *XWx-7A* so on. In this thesis, the symbols of *Wx-A1*, *Wx-B1* and *Wx-D1* which designate the A-, B- and D-genome members, respectively, are assigned since the wheat GBSS I genes are members of an homologous set over the three genome. Different alleles at a locus are designated by a lower case italic letter following the locus designation, so that *Wx-A1a* is the wild-type allele producing the Wx-A1 protein, whereas *Wx-A1b* is the null allele lacking the protein.

The basic symbol for DNA markers of unknown function should be '*X*', followed by a laboratory designator, a number that identifies the probe used to detect the locus, a hyphen, and the symbol for the chromosome in which the locus is located. The letters in the laboratory designator should be lower-case and all characters in the locus symbol should be italicized. For example, *Xpsr115-4A* designates an RFLP locus located in chromosome 4A detected with *P*lant *S*cience *R*esearch probe 115 of the John Innes Centre, UK. Oat and barley cDNA clones isolated at Cornell University have been designated with the prefixes CDO and BCD, respectively, and *cdo* and *bcd* are appropriately used as laboratory designators in symbols for loci detected with these clones. On a map of chromosome 4A, the gene symbol may be abbreviated by omission of the hyphen and chromosome designation, like *Xpsr115-4A* to *Xpsr115*.

QTLs are loci controlling quantitative traits whose allele classes do not exhibit discontinuous

variation or clear segregation patterns. They are identified by basic symbol '*Q*'. The '*Q*' should be followed by a trait designator, a period, a laboratory designator, a hyphen and symbol for the chromosome in which the QTL is located. The trait designator should consist of no more than four and preferably three letters, the first of which is capitalized. Different QTLs for the same traits that are identified in one chromosome should be assigned the same symbol except for the addition of a period and an Arabic numeral after the chromosome designation. All characters in the locus symbol should again be italicized. For example, *QEet.ocs-4A.1* and *QEet.ocs-4A.2* would designate two ear emergence time QTLs identified in chromosome 4A by our laboratory, *O*bihiro University of Agriculture and Veterinary Medicine, *C*rop *S*cience Department. On a map of chromosome 4A, these could be abbreviated as *QEet.ocs.1* and *QEet.ocs.2*.

1.8 References

Ainthworth CC, Clark J, Balsdon J (1993) Expression, organisation and structure of the genes encoding the waxy protein (granule-bound starch synthase) in wheat. Plant Mol Biol 22: 67-82

Baba T, Kimura K, Mizuno K, Etoh H, Ishida Y, Shida O, Arai Y (1991) Sequence conservation of the catalytic regions of amylolytic enzymes in maize branching enzyme-1. Biochem Biophys Res Commun 181: 87-94

Baba T, Nishihara M, Mizuno K, Kawasaki T, Shimada M, Kobayashi E, Ohnishi S, Tanaka K, Arai Y (1993) Identification, cDNA cloning, and gene expression of soluble starch synthase in rice (*Oryza sativa* L.) immature seeds. Plant Physiol 103: 565-573

Ball S, Guan H-P, James M, Myers A, Keeling P, Mouille G, Buljon A, Colonna P, Preiss J (1996) From glycogen to amylopectin : A model for the biosynthesis of the starch granule. Cell 86: 349-352

Blakeney AB (1996) Rice. In : Henry RJ and Kettlewell PS (eds) Cereal grain quality. Chapman & Hall, London, pp 55-76

Buleon A, Colonna P, Planchot V, Ball S (1998) Starch granules : structure and biosynthesis. Int J Biol Macromol 23: 85-112

Burton RA, Bewley JD, Smith AM, Bhattacharya MK, Tatge H, Ring S, Bull V, Hamilton WDO, Martin C (1995) Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. Plant J 7: 3-15

Chao S, Sharp PJ, Worland AJ, Warham EJ, Koebner RMD, Gale MD (1989) RFLP-based genetic maps of wheat homoeologous group 7 chromosomes. Theor Appl Genet 78: 495-504

Clark JR, Robertson MR, Ainthworth CC (1991) Nucleotide sequence of a wheat (*Triticum aestivum* L.) cDNA clone encoding the waxy protein. Plant Mol Biol 16: 1099-1101

Denyer K, Smith AM (1992) The purification and characterisation of two forms of soluble starch

synthase from developing pea embryos. Planta 186: 609-617

Denyer K, Barber LM, Burton R, Hedley CL, Hylton CM, Johnson S, Jones DA, Marshall J, Smith AM, Tatge H, Tomlinson K, Wang TL (1995a) The isolation and characterization of novel low-amylose mutants of *Pisum sativum* L. Plant Cell Env 18: 1019-1026

Denyer K, Hylton CM, Jenner CF, Smith AM (1995b) Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. Planta 196: 256-265

Devos KM, Dubcovsky J, Dvorak J, Chinoy CN, Gale MD (1995) Structural evolution of wheat chromosomes 4A, 5A, and 7B and its impact on recombination. Theor Appl Genet 91: 282-288

Doehlert DC, Knutson CA (1991) Two classes of starch debranching enzymes from developing maize kernels. J Plant Physiol 138: 566-572

Dry I, Smith AM, Edwards EA, Bhattacharyya M, Dunn P, Martin C (1992) Characterisation of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs. Plant J 2: 193-202

Echt C, Schwartz D (1981) Evidence for the inclusion of controlling elements within the structural gene at the *waxy* locus in maize. Genetics 99: 275-284

Edwards A, Marshall J, Sidebottom C, Visser RGF, Smith AM, Martin C (1995) Biochemical and molecular characterisation of a novel starch synthase from potato tubers. Plant Cell 8: 283-294

Edwards A, Fulton DC, Hylton CM, Jobling SA, Gidley M, Rossner U, Martin C, Smith AM (1999) A combined reduction in activity of starch synthases II and II of potato has novel effects on the starch of tubers. Plant J 17: 251-261

Fisher DK, Boyer CD, Hannah LC (1993) Starch branching enzyme II from maize endosperm. Plant Physiol 102: 61045-61046

Flipse E, Keetels CJAM, Jacobsen E, Visser RGF (1996a) The dosage effect of the wildtype GBSS allele is linear for GBSS activity but not for amylose : absence of amylose has a distinct influence on the physico-chemical properties of starch. Theor Appl Genet 92: 121-127

Flipse E, Suurs L, Keetels CJAM, Kossmann J, Jacobsen E, Visser RGF (1996b) Introduction of sense and antisense cDNA for branching enzyme in amylose-free potato mutant leads to physico-chemical changes in the starch. Planta 198: 340-347

Fujita S, Yamamoto H, Sugimoto Y, Morita N, Yamamori M (1998) Thermal and crystalline properties of waxy wheat (*Triticum aestivum* L.) starch. J Cereal Sci 27: 1-5

Gao M, Chibbar RN (1998) Molecular and biochemical characterization of soluble starch synthases in wheat kernels. In : Slinkard AE (ed) Proc 9th Int Wheat Genet Symp vol 4. Univ Exten Press, Univ of Saskatchewan, Canada, pp 151-153

Graybosch RA (1998) Waxy wheat : Origin, properties, and prospects. Trends Food Sci Technology 9: 135-142

Hayakawa K, Tanaka K, Nakamura T, Endo S, Hoshino T (1997) Quality characteristics of waxy

hexaploid wheat (*Triticum aestivum* L.) : Properties of starch gelatinization and retrogradation. Cereal Chem 74: 576-580

Hovenkamp-Hermelink JHM, Jacobson E, Ponstein AS, Visser RGF, Vos-Scheperkeuter GH, Bijmolt EW, de Vries JN, Witholt B, Feenstra WJ (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). Theor Appl Genet 75: 217-221

Jenner CF (1994) Starch synthesis in the kernel of wheat under high temperature conditions. Aust J Plant Physiol 21: 791-806

Kawasaki T, Mizuno K, Baba T, Shimada H (1993) Molecular analysis of the gene encoding a rice starch branching enzyme. Mol Gen Genet 237: 10-16

Keeling PL, Wood JR, Tyson RW, Bridges IG (1988) Starch biosynthesis in developing wheat grain. Evidence against the direct involvement of triose phosphates in metabolic pathway. Plant Physiol 78: 311-319

Li Z, Rahman S, Kosar-Hashemi B, Mouille G, Appels R, Morell MK (1999) Cloning and characterization of a gene encoding wheat starch synthase I. Theor Appl Genet 98: 1208-1216

Martin C, Smith AM (1995) Starch biosynthesis. Plant Cell 7: 971-985

McIntosh RA, Hart GE, Devos KM, Gale MD, Rogers WJ (1998) Catalogue of gene symbols for wheat. In : Slinkard AE (ed) Proc 9th Int Wheat Genet Symp vol 5. Univ Exten Press, Univ of Saskatchewan, Canada.

McKenzie KS, Rutger JN (1983) Genetic analysis of amylose content, alkali spreading score, and grain dimensions in rice. Crop Sci 23: 306-311

Mizuno K, Kawasaki T, Shimada H, Satoh H, Kobayashi E, Okumura S, Arai Y, Baba T (1993) Alteration of the structural properties of starch components by the lack of an isoform of starch branching enzyme in rice seeds. J Bio Chem 268: 19084-19081

Morell MK, Rahman S, Abrahams SL, Appels R (1995) The biochemistry and molecular biology of starch synthesis in cereals. Aust J Plant Physiol 22: 647-660

Morell MK, Blennow A, Kosar-Hashemi B, Samuel MS (1997) Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. Plant Physiol 113: 201-208

Morris CF (1998) Genetic determinants of wheat grain quality. In : Slinkard AE (ed) Proc 9th Int Wheat Genet Symp vol 1. Univ Exten Press, Univ of Saskatchewan, Canada, pp 245-253

Morrison WR, Gadan H (1987) The amylose and lipid contents of starch granules in developing wheat endosperm. J Cereal Sci 5:263-275

Mu C, Harm C, Ko YT, Singletary GW, Keeling PL, Wasserman BP (1994) Association of a 76 kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm. Plant J 6: 151-159

Murai J, Taira T, Ohta D (1999) Isolation and characterization of the three *Waxy* genes encoding the

granule-bound starch synthase in hexaploid wheat. Gene 234: 71-79

Nair RB, Baga M, Scoles GJ, Kartha KK, Chibbar RN (1997) Isolation, characterization and expression analysis of a starch branching enzyme II cDNA from wheat. Plant Sci 122: 153-163

Nakamura T, Yamamori M, Hirano H, Hidaka S (1993) Identification of three Wx proteins in wheat (*Triticum aestivum* L.). Biochem Genet 31: 75-86

Nakamura T, Yamamori M, Hirano H, Hidaka S, Nagamine T (1995) Production of waxy (amylose-free) wheats. Mol Gen Genet 248: 253-259

Nakamura T, Vrinten P, Hayakawa K, Ikeda J (1998) Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. Plant Physiol 118: 451-459

Nalanjo T, Roca A, Goicoecha PG, Giraldez R (1987) Arm homoeology of wheat and rye chromosomes. Genome 29: 873-882

Nelson OE, Chourey PS, Chang MT (1978) Nucleoside diphosphate sugar-starch glucosyl transferase activity of *wx* starch granules. Plant Physiol 62: 383-386

Pan D, Nelson OE (1984) A debranching enzyme deficiency in endosperms of the *sugary-1* mutants of maize. Plant Physiol 74: 324-328

Poulsen P, Keiberg JD (1993) Starch branching enzyme cDNA from *Solanum tuberosum*. Plant Physiol 102: 1053-1054

Preiss J, Levi C (1980) Starch biosynthesis and degradation. In: Preiss J (ed) Carbohydrates : structure and function. The Biochem Plants 3. Academic Press Inc, pp 371-369

Rahman S, Kosar-Hashemi B, Samuel MS, Hill A, Abbot DC, Skerritt JH, Preiss J, Appels R, Morell MK (1995) The major proteins of wheat endosperm starch granules. Aust J Plant Physiol 22: 793-803

Rahman S, Abrahams S, Abbott D, Mukai Y, Samuel M, Morell M, Appels R (1997) A complex arrangement of genes at a starch branching enzyme I locus in D-genome donor of wheat. Genome 40: 465-474

Rahman S, Li Z, Abrahams S, Abbott D, Appls R, Morell M (1999) Characterisation of a gene encoding wheat endosperm starch branching enzyme-1. Theor Appl Genet 98: 156-163

Repellin A, Nair RB, Baga M, Chibaar RN (1997) Isolation of a starch branching enzyme I cDNA from a wheat endosperm library. (Accession no Y12320). Plant Gene Resister PGR 97-094 : http://www.tarweed.com/pgr/PGR 97-094.html

Rhode W, Becker D, Salamini F (1988) Structural analysis of the *waxy* locus from *Hordeum vulgare*. Nucleis Acids Res 16: 7185-7186

Sano Y, Katsumata M, Okuno K (1986) Genetic studies of speciation in cultivated rice. 5. Inter- and intraspecific differentiation in the *waxy* gene expression of rice. Euphytica 35: 1-9

Schofield JD, Greenwell P (1987) Wheat starch granule proteins and their technological significance. In : Morton ID (ed) Cereals in a European context. Ellis Horwood, Chichester, UK, pp 399-420

Shannon JC, Garwood DL (1984) Genetics and physiology of starch development. In: Whistler RL, BeMiller JN, Paschall EF (eds) Starch : chemistry and technology, 2nd edn. Academic Press, Orland, USA, pp 25-86

Sharp PJ (1997) Homoeologous group 2. In: McGuire PE, Qualset CO (eds) Progress in genome mapping of wheat and related species. Joint Proc 5th 6th Public Workshops Int Triticeae Mapping Initiative. Genetic Resources Conservation Program, Univ of California, pp 24-37

Shimada H, Tada Y, Kawasaki T, Fujimura T (1993) Antisense regulation of the rice waxy gene expression using a PCR-amplified fragment of the rice genome reduces the amylose content in grain starch. Theor Appl Genet 86: 665-672

Smith AM (1988) Major differences in isoforms of starch-branching enzyme between developing embryos of round- and wrinckled-seeded peas (*Pisum sativum* L.). Planta 175: 270-279

Smith AM (1999) Making starch. Curr Opn Plant Biol 2: 223-229

Smith AM, Denyer K, Martin CR (1995) What controls the amount and structure of starch in storage organs. Plant Physiol 107: 673-677

Smith AM, Denyer K, Martin C (1997) The synthesis of the starch granule. Annu Rev Plant Physiol Plant Mol Biol 48: 67-87

Stark JR, Lynn A (1992) Starch granules large and small. Biochem Soc Transact 20: 7-12

Takaoka M, Watanabe S, Sassa H, Yamamori M, Nakamura T, Sasakuma T, Hirano H (1997) Structural characterization of high molecular weight starch granule-bound proteins in wheat (*Triticum aestivum* L.). J Agric Food Chem 45: 2929-2934

Tetlow IJ, Blissett KJ, Emes MJ (1994) Starch synthesis and carbohydrate oxidation in amyloplasts from developing wheat endosperm. Planta 194: 454-460

Tsai CY (1974) The function of the *waxy* locus in starch synthesis in maize endosperm. Biochem Genet 11: 83-96

Tyynela J, Schulman AH (1993) An analysis of soluble starch synthase isozymes from the developing grains of normal and *shx* cv. Bomi barley (*Hordeum vulgare*). Physiol Plant 89: 835-841

Visser RGF, Jacobsen E (1993) Toward modifying plants for altered starch content and composition. Trends Biotech 11: 63-68

Visser RFG, Somhorst I, Kuipers GJ, Ruys NJ, Feenstra WJ, Jacobsen E (1991) Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. Mol Gen Genet 225: 289-296

Vrinten P, Nakamura T, Yamamori M (1999) Molecular characterization of *waxy* mutations in wheat. Mol Gen Genet 261: 463-471

Wang Z, Zeng F, Shen G, Gao J, Snustad D, Li M, Zhang J, Hong M (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy* gene. Plant J 7:

613-622

Watanabe N, Noda Y, Goto N, Miura H (1998) Variation for apparent amylose content of endosperm starch in *Triticum durum* and *Aegilops squarrosa*. Euphytica 101:283-286

Wrigley CW, Morris CF (1996) Breeding cereals for quality improvement. In : Henry RJ, Kettlewell PS (eds) Cereal grain quality. Chapman & Hall, London, pp 321-370

Yamamori M, Endo TR (1996) Variation of starch granule proteins and chromosome mapping of their coding genes in common wheat. Theor Appl Genet 93: 275-281

Yamamori M, Fujita S, Hayakawa K, Matsuki J, Yasui T (2000) Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. Theor Appl Genet 101:21-29

Zeng M, Morris CF, Batey IL, Wrigley CW (1997) Sources of variation for starch gelatinization, pasting, and gelation properties in wheat. Cereal Chem 74: 63-71

Zobel HF (1984) Gelatinization of starch and mechanical properties of starch pastes. In : Whistler RL, BeMiller JN, Paschall EF (eds) Starch : chemistry and technology, 2nd edn. Academic Press, Orland, pp 285-309

Table 1. Enzymes involved in starch synthesis in the wheat endosperm

Enzymes	Molecular weight	Fraction	Location of the genes	References
GBSS	60 kDa	granule-bound	7AS, 4AL, 7DL	Nakamura et al. (1993)
SSS (SGP-3)	75 kDa	soluble and granule-bounnd	7AS, 7BS, 7DS	Yamamori and Endo (1996)
SBE (SGP-3)	85 kDa	soluble and granule-bound	Group 7 (wSBE I) Group 2 (wSBE II)	Morell et al. (1997) Rahman et al. (1999) Sharp (1997)
SGP-1	100-115 kDa	soluble and granule-bound	7AS, 7BS, 7DS	Denyer et al. (1995) Yamamori and Endo (1996)

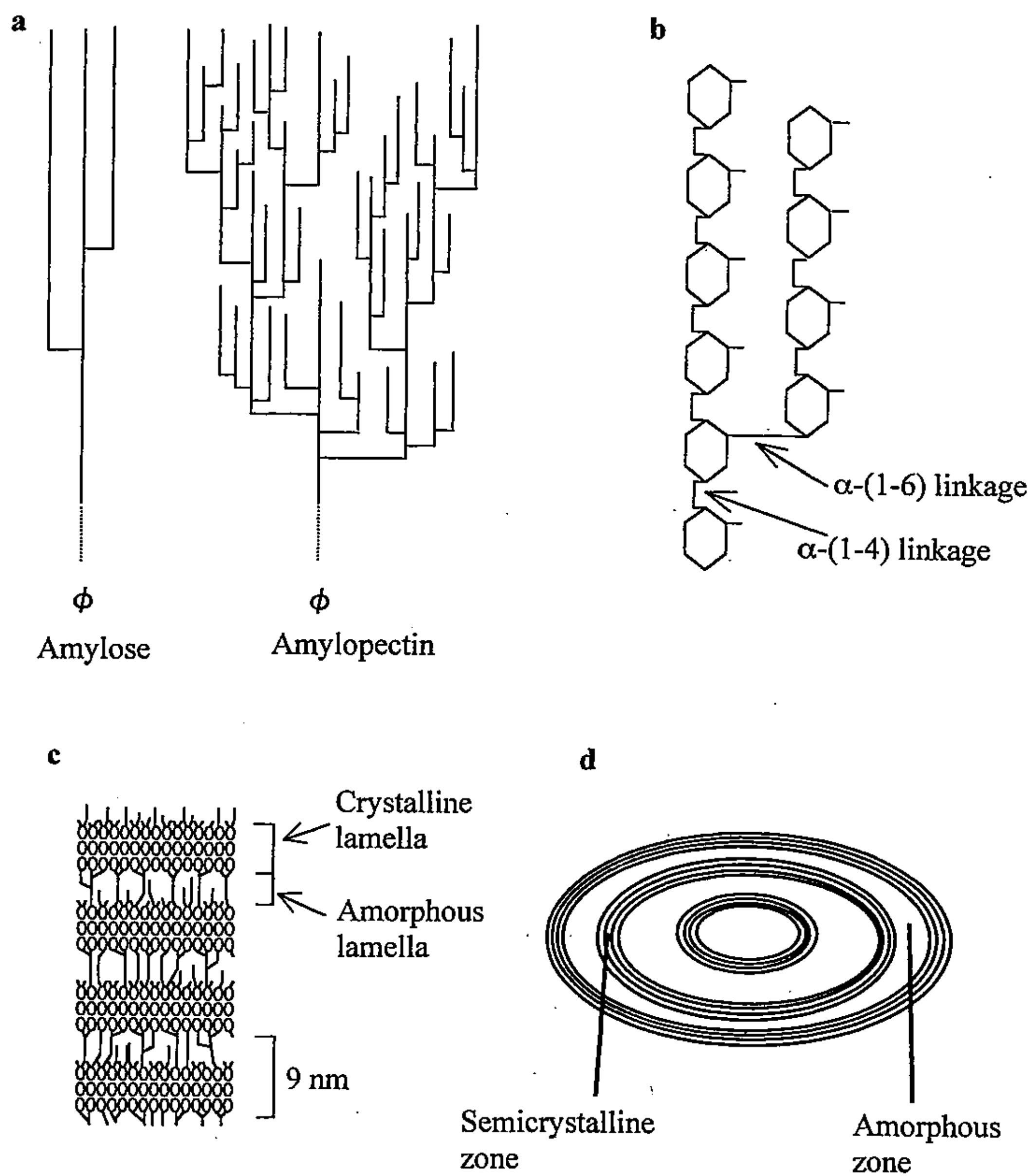


Fig. 1. Structure of starch granule.
(a) ; Amylose and amylopectin, (b) ; α -(1-4) linkage and α -(1-6) linkage, (c) ; Arrangement of clusters to form alternating crystalline and amorphous lamellae, (d) ; Slicing starch granule, consisting of semiclystaline and amorphous lamellae.

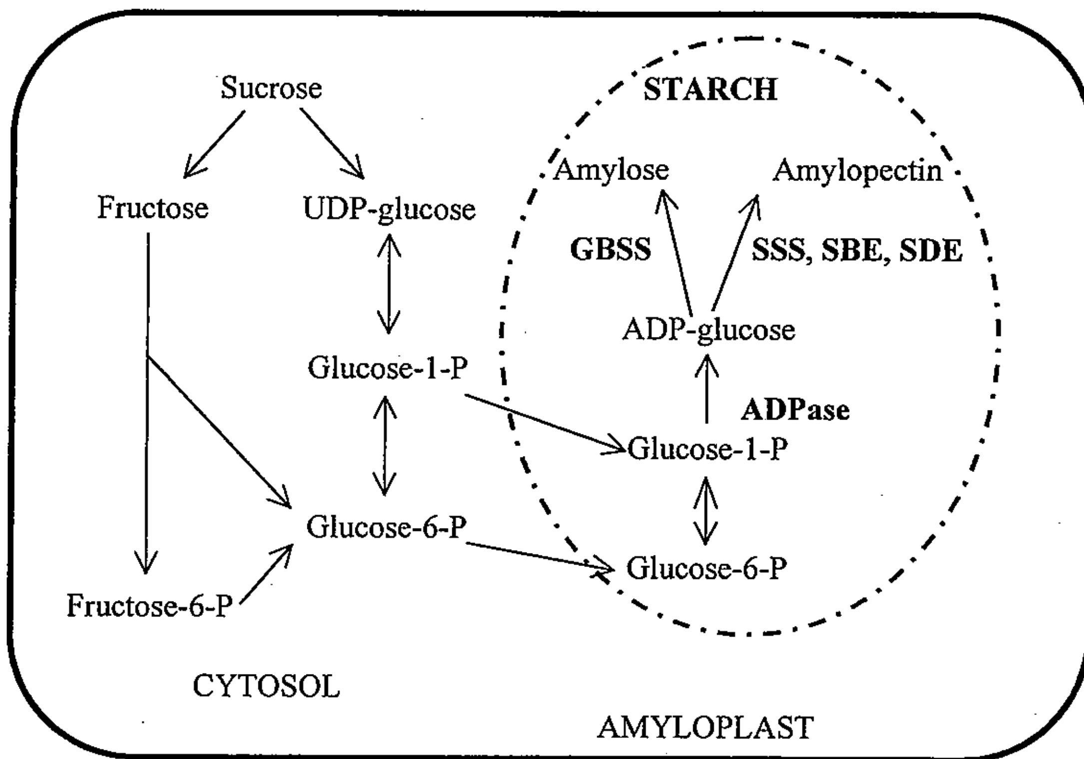


Fig. 2. Pathway of starch biosynthesis in amyloplast of cereal endosperm.

ADPase; ADP-glucose pyrophospholyase

GBSS; granule-bound starch synthesis

SSS; soluble starch synthase

SBE; starch branching enzyme

SDE; starch debranching enzyme

Chapter 2. Amylose synthesis capacity of the three *Wx* genes of cv. Chinese Spring

Abstract

The *Wx* locus controls amylose synthesis in the cereal endosperm. Hexaploid wheat (*Triticum aestivum* L.) has the three *Wx* loci on chromosomes 7A (*Wx-A1*), 4A (*Wx-B1*) and 7D (*Wx-D1*). To verify the effects of null alleles on reducing amylose content and determine the amylose synthesis capacity of each *Wx* gene independently and accurately, we produced eight possible types of recombinant lines carrying different null alleles at the *Wx* loci under the 'Chinese Spring' genetic background. Amylose content varied from 0% of the waxy 'Chinese Spring' to 25% of the 'Chinese Spring' normal type. The reducing effect of the single null alleles was the largest in *Wx-B1b,* and there was no significant difference between *Wx-A1b* and *Wx-D1b*. More than 3% reductions in amylose content were detected in the double null types. The results of the double null lines further demonstrated that for the capacity of amylose synthesis, *Wx-B1a* predominates and produces 21-22% amylose, followed by *Wx-D1a* (20-21%) and *Wx-A1a* (15-18%). These significant differences were partly correlated with variation in the amounts of the Wx proteins produced by different *Wx* genes. However, comparisons of the double null lines with the single null or normal lines indicated that amylose content was not linearly proportional to the number of the *Wx* genes, suggesting that the three *Wx* genes act in an epistatic manner.

Key words *: Triticum aestivum*, amylose content, granule-bound starch synthase, null alleles, *Wx* loci

Introduction

Starch is the major constituent of the cereal endosperm. The starch granule usually comprises two different forms of polymers of glucose, amylose and amylopectin, which differ in the degree of branching of a linear α -1,4 glucose backbone. Amylopectin is much more branched than amylose. The starch reserves of plants usually consist of about 20-30% amylose and 70-80% amylopectin (Preiss, 1991). The granule-bound starch synthase with molecular weight of 60 kDa, GBSS I or the so-called Wx protein, is the key enzyme involved in amylose synthesis of endosperm starch and is the product of genes at the *Wx* loci (Tsai, 1974; Echt and Schwartz, 1981).

In hexaploid wheat (*Triticum aestivum* L.), the three *Wx* loci which are organized as a triplicate set of single-copy homoeoloci are located on chromosomes 7A (*Wx-A1*), 4A (*Wx-B1*) and 7D (*Wx-D1*) (Chao et al., 1989; Miura et al., 1994). Low-amylose-content cultivars are preferred for the noodle manufactory (Yamamori et al., 1992; Miura and Tanii, 1994) and the potential use of starch with reduced amylose content is a current topic of discussion among wheat breeders and geneticists. Null, or non-functional,

alleles are now known from all three loci (Yamamori et al., 1994). The segregation of the null alleles at the *Wx* loci will be significant in the management of breeding programs that aim to introgress the null alleles into established cultivars and advanced lines.

Using aneuploid lines and single-chromosome substitution lines, we have demonstrated that the three *Wx* genes are not similar in their effects to modify amylose content as the null *Wx-B1b* allele provides the largest reduction in the content through the lack of the Wx-B1 protein (Miura et al., 1994; Miura and Sugawara, 1996). However, the amylose synthesis ability of each *Wx* gene has not been defined well, because of the lack of double null genotypes. We have produced eight possible types of homozygous recombinant lines having different null alleles at the *Wx* loci under the 'Chinese Spring' genetic background, and investigated variation for amylose content of starch deposited in the endosperm. This makes it possible to verify the effects of null alleles on reducing amylose content and determine the amylose synthesis capacity of each *Wx* gene independently and accurately.

Materials and methods

Plant material

'Chinese Spring' (CS) carries the *Wx-A1a*, *Wx-B1a* and *Wx-D1a* alleles and thus can produce all of the three Wx proteins. Using the conventional procedures described by Law and Worland (1973), monotelosomics for chromosomes 7A and 4A of CS as the recurrent parents were pollinated by the Japanese spring wheat 'Kanto 107' carrying the null *Wx-A1b* and *Wx-B1b* alleles. Eleven backcrosses of the monosomic substitutions to the recurrent monotelosomics were performed and then disomic lines were extracted after selfing. Similarly, introgression of the null *Wx-D1b* from the Chinese spring wheat 'Bai Huo' into the monotelosomic 7D of CS was carried out through eight backcrosses. The resultant three single-chromosome substitution lines, designated as CS*11/Kanto107 7A, CS*11/Kanto107 4A and CS*8/Bai Huo 7D, were checked for their validity of substitution by electrophoretic phenotypes of the Wx proteins (Miura and Sugawara, 1996).

Subsequently, F_1 plants from the intercrosses between CS and the substitution lines were used to produce homozygous recombinant lines carrying single null allele sets at the *Wx* loci. Anther culture method was employed to produce doubled haploid lines. The three types of double null lines were obtained from the F_7 recombinant inbred lines which were produced from the crosses between the substitution lines by single-seed descent. The amylose-free or waxy CS was selected from the F_2 progeny derived from CS*11/Kanto107 7A / CS*11/Kanto107 4A // CS*8/Bai Huo7D and backcrossed twice to the normal CS. The eight types of the Wx-protein deficient lines developed include sib-lines which were expected to be homozygous recombinant lines for the substituted chromosomes carrying the *Wx* loci and have an identical genetic background of CS. Variation between lines within the types would permit not only to assess effects of the genetic background but to provide a measure of differences ascribable to

segregated genes independent of *Wx* in the substituted chromosomes (Law and Worland, 1973).

Electrophoresis

The lines produced were typed for the null *Wx* alleles using the single dimension SDS-PAGE. Starch granule preparation and electrophoresis were performed as described by Nakamura et al. (1993), with the modification that a 15 % SDS polyacrylamide gel with an acrylamide/BIS concentration of 30:0.135 was used for electrophoresis.

Experiment

Variation for amylose content was evaluated in the two experiments. Twenty-two lines including CS as a standard were grown and harvested in the experimental field and a glasshouse of Obihiro University of Agriculture and Veterinary Medicine in the 1997 growing season. Each line planted in the field experiment was represented by a single plot of 12 plants, spaced 10 cm between plants within a row and 30 cm between rows. In the glasshouse experiment, lines were grown in 24 cm-plots containing compost. Six plants per line were raised.

In each experiment, 80 g of grain samples per line were conditioned to about 14 % moisture content and were milled on a Brabender Quadrant Junior Test Mill to produce a 60 % extraction flour. Preparation of starch and evaluation of amylose content were conducted as described by Miura et al. (1994). The amylose content per 100 mg of starch granules was colorimetrically determined with iodine using the Auto Analyzer System II (Bran + Lubbe Co.). Each assay was carried out at least three times.

Student's t-tests were used for one-environment data to detect significant differences between CS and each of the Wx-protein deficient lines. For the two-environment data, analysis of variance was performed to partition the line variation into comparisons between and within the eight types where the experimental error was estimated from the mean square of line x environment interaction.

Results and discussion

To confirm the alleles at the *Wx* loci, the electrophoretic phenotypes of the Wx proteins were assessed. As expected, eight types of the Wx-protein phenotype were observed (Fig. 1). The waxy or amylose-free type 8 (lane 8) produced no Wx proteins and this was confirmed with iodine staining of the cut endosperm surfaces. Comparisons of the three double null types (lanes 5-7) showed that the apparent amount of the Wx-A1 protein was the smallest, followed by the Wx-D1 and Wx-B1 proteins.

The results of amylose content are summarized in Table 1. CS produced around 25% amylose over two environments. As anticipated, the waxy CS (wxABD) did not synthesize amylose. In the remaining six types of Wx-protein deficient lines (types 2–7), amylose content was distributed between these two extremes. Analysis of variance indicated that highly significant line differences ($P<0.001$) and

no significant effect of environments. When this genetic variation was partitioned, it was found that the line differences were greatly attributable to the differences between the eight types (data not shown). Except type 7, the sib-lines within types produced almost identical amylose contents to each other, revealing negligible effects of the genetic background.

For the single null alleles, type 3 (wxB) having the null *Wx-B1b* and lacking the Wx-B1 protein showed more than 2% lower amylose content than CS. Amylose content in type 2 (wxA) was almost similar to that in type 4 (wxD), indicating that the null *Wx-A1b* was comparable to the null *Wx-D1b* with respect to the effect on the amylose reduction. Consequently, the reducing effect of the single null allele was shown to be the largest in *Wx-B1b*, and there was no significant difference between *Wx-D1b* and *Wx-A1b*. These confirmed our previous results derived from aneuploid lines available in CS (Miura et al., 1994; Miura and Sugawara, 1996) and from recombinant substitution lines (Araki et al., 1999).

In the lines having double null alleles, more than 3% reductions in amylose content were detected. Type 7 (wxBD) which carries the *Wx-A1a*, *Wx-B1b* and *Wx-D1b* alleles and produces only the Wx-A1 protein showed the lowest amylose content of around 15-18 %, followed by type 5 (wxAB) lacking the Wx-A1 and Wx-B1 proteins and type 6 (wxAD) lacking the Wx-A1 and Wx-D1 proteins. The lowest amylose content in type 7 also means that if the Wx-B1 protein lacks, decrease in amylose is greatly accelerated by deficiency of the Wx-D1 protein, rather than deficiency of the Wx-A1 protein. Furthermore, in type 7, variation was detected between wxBD-3 and the other two lines. This variation might be partly explained by the segregation of independent loci, since a QTL affecting amylose content has been identified on the short arm of chromosome 4A and there is an allelic difference at this QTL between the parents (Araki et al., 1999). This type was also sensitive to environmental changes, as amylose content in the three sib-lines was significantly lower in the glasshouse-grown plants than in the field-grown plants.

The results of the double null types further demonstrated that for the capacity of amylose synthesis, *Wx-B1a* predominates and produces 21-22% amylose, and there is a significant difference between *Wx-D1a* (20-21%) and *Wx-A1a* (15-18%). As the apparent amount of the Wx-B1 protein was the largest, followed by the Wx-D1 and Wx-A1 proteins (Fig. 1), the effects of the different *Wx* genes on amylose content could be at least partly explained by variation in the amount of Wx proteins.

Recently nucleotide sequences of the three genomic *Wx* genes of CS encoding the Wx proteins have been determined (Murai et al., 1999). Very high levels of homology in exon areas seem to be hard to explain the large differences in amylose synthesis capacity among the three *Wx* genes. Compared with the protein-coding regions, promotor regions responsible for gene regulation have not always been conserved among organisms, even among closely related species.

In cultivated rice, there are two wild-type alleles, Wx^a and Wx^b. Wx^a is characteristic of Indica rice and Wx^b is found mainly in Japonica rice. Consistent with the high levels (about 10-hold) of the *Wx*

transcript and Wx protein in *Wx*^{a}, the amylose content in Indica rice is higher than in Japonica rice (Sano, 1984; Sano et al., 1985). While there is only one amino acid difference between *Wx*^{a} and *Wx*^{b} gene products, it is likely that their specific activities are similar (Hirano and Sano, 1991; Okagaki 1992). To explain this conflict, it has been pointed out that variation in the amounts of the Wx protein and amylose content in Japonica and Indica types of rice is regulated at the level of *Wx* transcript processing and, more specifically, at the stage of intron I excision from the *Wx* pre-mRNA (Okagaki, 1992; Wang et al., 1995). Hirano et al. (1998) and Isshiki et al. (1998) have demonstrated that GT sequence at the 5' splice junction of intron I is essential for the high expression level in *Wx*^{a} which can produce a larger amount of the Wx protein and a higher amylose content than in *Wx*^{b}. This is supported by the relation between the polymorphism at 5' splice junction and amylose content by analyzing 92 U.S. rice cultivars (Ayres et al., 1997). In wheat, whether the efficiency of excision of introns in each *Wx* gene associates with differences in amylose content is not determined, thus it would be of interest to examine this respect using the double null lines.

From comparisons of the double null types with the single null or normal types, it was found that the amount of the Wx proteins was correlated to the number of *Wx* genes. However, amylose content was not additively increased by accumulation of the functional *Wx* alleles. For example, under the field conditions, type 7 carrying *Wx-A1a* produced 18.10% of mean amylose content and type 6 carrying *Wx-B1a* produced 21.43 %, but the mean amylose content of type 4 carrying *Wx-A1a* plus *Wx-B1a* was 23.14%. Hence it appears that a certain level of activity of the single *Wx* gene, or else of the Wx protein, led to a considerable amount of amylose, so that further increases in the different *Wx* gene products bring about only a small amount of additional raise in amylose content. This suggests an epistatic manner of the three *Wx* genes, reducing or inhibiting amylose synthesis when at least two genes produce the Wx proteins. A similar finding, that there is no linear correlation between the Wx protein activity and amylose content, has been reported in maize (Tsai, 1974; Echt and Schwartz, 1981), rice (Sano et al., 1985) and potato (Flipse et al., 1996).

A complete association between the presence of the null *Wx-B1b* allele and the classification of the white salted noodle category in Australian and Japanese cultivars has been reported (Miura and Tanii, 1994; Yamamori et al., 1994; Zhao et al., 1998). In addition, as mentioned earlier, the amylose-reducing effect of the single null allele was larger in *Wx-B1b* than *Wx-D1b* and *Wx-A1b*. These evidence demonstrate that the null *Wx-B1b* allele appears to determine a potential for producing noodle quality flour. Furthermore, the work reported here indicates that introgression of a null allele set of *Wx-B1b* and null *Wx-D1b* result in a striking reduction by about 15 % amylose as found in the glasshouse-grown wxBD-3 (Table 1). Production of adapted double null genotypes having this allele set would be expected as the more likely consistent source of low amylose starch. So it is significant to know if such a variation, as well as waxy wheat, contributes toward diverse end-use properties of wheat flour.

References

Araki E, Miura H, Sawada S (1999) Identification of genetic loci affecting amylose content and agronomic traits on chromosome 4A of wheat. Theor Appl Genet 98: 977-984

Ayres NM, McClung AM, Larkin PD, Bligh HFJ, Jones CA, Park WD (1997) Microsatellites and a single-nucleotide polymorphism differentiate apparent amylose classes in an extended pedigree of US rice germplasm. Theor Appl Genet 94 : 773-781.

Chao S, Sharp PJ, Worland AJ, Warham EJ, Koebner RMD, Gale MD (1989) RFLP-based genetic maps of wheat homoeologous group-7 chromosomes. Theor Appl Genet 78: 495-504.

Echt CS, Schwartz D (1981) Evidence for the inclusion of controlling elements within the structural gene at the waxy locus in maize. Genetics 99 : 275-284.

Flipse E, Keetels CJAM, Jacobsen E, Visser RGF (1996) The dosage effect of the wildtype GBSS allele is linear for GBSS activity but not for amylose content : absence of amylose has a distinct influence on the physico-chemical properties of starch. Theor Appl Genet 92:121-127.

Hirano H-Y, Eiguchi M, Sano Y (1998) A single base change altered the regulation of *waxy* gene at the posttranscriptional level during the domestication of rice. Mol Biol Evol 15 : 978-987.

Isshiki M, Morino K, Nakajima M, Okagaki RJ, Wessler SR, Izawa T, Shimamoto K (1998) A naturally occuring functional allele of the rice *waxy* locus has a GT to TT mutation at the 5' splice site of the first intron. Plant J 15: 133-138.

Law CN, Worland AJ (1973) Aneuploidy in wheat and its uses in genetic analysis. Plant Breeding Inst Annu Rep 1972. pp. 25-65

Miura H, Tanii S (1994) Endosperm starch properties in several wheat cultivars preferred for Japanese noodles. Euphytica 72 :171-175.

Miura H, Sugawara A (1996) Dosage effects of the three *Wx* genes on amylose synthesis in wheat endosperm. Theor Appl Genet 93: 1066-1070.

Miura H, Tanii S, Nakamura T, Watanabe N (1994) Genetic control of amylose content in wheat endosperm starch and differential effects of three *Wx* genes. Theor Appl Genet 89: 276-280.

Murai J, Moriyama Y, Taira T (1998) Phylogenetic analysis based on the nucleotide sequences of the *waxy* genes in polyploid wheats and related diploid species. In: Slinkard AE (ed.), Proc 9th Wheat Genet Symp 2, pp. 95-97. Univ. Exten. Press, Univ. of Saskatchewan, Canada.

NakamuraT, Yamamori M, Hirano H, Hidaka S (1993) Identification of three Wx proteins in wheat (*Triticum aestivum* L.). Biochem Genet 31: 75-86.

Okagaki RJ (1992) Nucleotide sequence of a long cDNA from the rice *waxy* gene. Plant Mol Biol 19:513-516.

Preiss J (1991) Biology and molecular biology of starch synthesis and its regulation. In: Morton ID (ed.), Oxford Surv Plant Mol Cell Biol 7, pp. 59-114. Oxford Univ. Press, UK.

Sano Y (1984) Differential regulation of waxy gene expression in rice endosperm. Theor Appl Genet 68:467-473.

Sano Y, Katsumata M, Amano E (1985) Correlation between the amount of amylose and Wx protein in rice endosperm. SABRAO J 17:121-127.

Tsai CY (1974) The function of the waxy locus in starch synthesis in maize endosperm. Biochem Genet 11:83-96.

Wang Z, Zeng F, Shen G, Gao J, Snustad D, Li M, Zhang J, Hong MM (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy* gene. Plant J 7: 613-622.

Yamamori M, Nakamura T, Kuroda A (1992) Variations in the content of starch-granule bound protein among several Japanese cultivars of common wheat (*Triticum aestivum* L.). Euphytica 64:215-219.

Yamamori M, Nakamura T, Endo TR, Nagamine T (1994) Waxy protein deficiency and chromosome location of coding genes in common wheat. Theor Appl Genet 89: 179-184.

Zhao X, Batey I, Sharp PJ, Crosbie G, Barclay I, Wilson R, Morell M, Appels R 1998. A single genetic locus associated with starch granule properties and noodle quality in wheat. J Cereal Sci 27: 7-13.

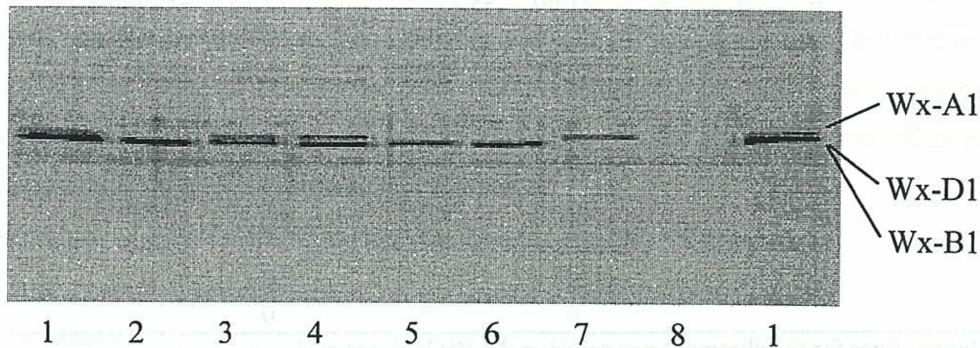


Fig. 1. Electrophoretic phenotypes in the eight possible types of the Wx-protein deficient lines. Lanes from 1 to 8 correspond to the genotypes shown in Table 1. Lane 1 : the wild type producing all of the three Wx proteins; lanes 2, 3 and 4 : single null types lacking Wx-A1, Wx-B1 and Wx-D1 proteins respectively; lanes 5, 6 and 7 : double null types lacking Wx-A1 plus Wx-B1, Wx-A1 plus Wx-D1, Wx-B1 plus Wx-D1 proteins respectively; lane 8 : waxy or amylose-free type, lacking all of the three Wx proteins

Table 1. Amylose content and its deviation from CS in the eight types of the Wx-protein deficient lines grown in two environments

Genetic lines		Wx proteins			Amylose content (%)			
		A1	B1	D1	Field		Glasshouse	
1	CS	+	+	+	24.88		24.91	
	CS-1[a]				24.49	-0.39	24.79	-0.13
	CS-2				24.81	-0.07	24.38	-0.53
2	CS*11/Kanto107 7A	–	+	+	23.60	-1.27*	23.58	-1.33**
	wxA-1				23.10	-1.78*	23.52	-1.40**
	wxA-2				23.66	-1.22*	22.59	-2.33*
3	CS*11/Kanto107 4A	+	–	+	22.89	-1.99***	21.93	-2.98***
	wxB-1				22.46	-2.42*	22.97	-1.94*
	wxB-2				22.64	-2.24*	22.41	-2.50**
4	CS*8/Bai Huo 7D	+	+	–	23.00	-1.88**	23.50	-1.42**
	wxD-1				23.29	-1.59*	23.35	-1.56**
5	wxAB-1	–	–	+	20.08	-4.79***	19.71	-5.20**
	wxAB-2				20.83	-4.05***	19.48	-5.43**
	wxAB-3				20.28	-4.60***	20.56	-4.35**
6	wxAD-1	–	+	–	21.60	-3.27**	21.13	-3.78***
	wxAD-2				21.75	-3.13**	22.79	-2.12*
	wxAD-3				20.93	-3.95***	21.14	-3.78***
7	wxBD-1	+	–	–	18.15	-6.73***	16.02	-8.89***
	wxBD-2				19.62	-5.26***	16.10	-8.82**
	wxBD-3				16.50	-8.38***	15.32	-9.59***
8	wxABD-1	–	–	–	0		0	
	wxABD-2				0		0	

[a] Homozygous recombinant lines for the chromosomes carrying the *Wx* loci, see text.

*, **, *** : Significant from CS at 0.05, 0.01, 0.001% levels, respectively.

Chapter 3. Development of near-isogenic lines carrying different null *Wx* alleles and their starch property

Abstract

The granule-bound starch synthase (GBSS I) encoded by the *Wx* genes, is involved in amylose synthesis. For analyses of mechanisms of amylose synthesis and associated starch properties in hexaploid wheat, eight possible genotypes having different combinations of the three null alleles at the *Wx* loci with a common genetic background are prerequisite. A near-isogenic population of doubled haploid (DH) lines was produced from Chinese Spring x the waxy Chinese Spring F_1 plants using the wheat x maize method. The Wx protein phenotypes of the DH progeny were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and found that the null alleles at each of the three *Wx* loci segregated in a Mendelian fashion. Field trial demonstrated no differences between the eight types for ear emergence time, plant height and grain yield traits. Amylose content in the endosperm starch was highest in the wild type while lowest in the waxy type having no Wx proteins. Comparison between single null types and double null types indicated that the amylose synthesis capacity of *Wx-A1a* allele is the lowest. Pasting properties of starch are the highest in the waxy type, followed by the double null types. Consequently, both peak viscosity and breakdown were negatively correlated with amylose content. The chain-length distribution analysis of amylopectin structure revealed no clear difference among the eight types, suggesting that the reduced GBSS I activity due to introgression of the null *Wx* alleles does not affect both the chain length and the degree of branching of amylopectin.

Key words: amylose content, amylopectin, doubled haploid, *Wx* gene, wheat

Introduction

Starch, the predominant reserve carbohydrate found in plants, plays a key role in human activities as a source of nutrition for humans and animals, and as a raw material for many non-food industries. Demand for many kinds of natural starches that possess novel physical and chemical properties is increasing, due to the desire to develop new starch products and to reduce the need for post harvest chemical modification processes. The amylose/amylopectin ratio of starch is extremely important in producing marketable flour products, because it affects the quality of end-uses (Moss, 1980; Oda et al., 1980; Toyokawa et al., 1989).

The granule-bound starch synthase (GBSS I), known as the Wx protein with a molecular weight of about 60 kDa, is the key enzyme involved in amylose synthesis. The Wx proteins in hexaploid wheat (*Triticum aestivum*) are encoded by three different *Wx* genes at a triplicate set of single-copy homoeoloci on chromosome arms of 7AS (*Wx-A1*), 4AL (*Wx-B1*) and 7DS (*Wx-D1*)

(Chao et al., 1989). The wild type can produce all of the three Wx proteins by the active *Wx-A1a, Wx-B1a* and *Wx-D1a* alleles, while the waxy or amylose free genotypes lack any Wx proteins due to null alleles at the three loci (Nakamura et al., 1995). The *Wx* genes do not solely control amylose content. Our recent study has detected a quantitative trait locus (QTL) with minor effects on amylose content in the short arm of chromosome 4A (Araki et al., 1999). Amylopectin structure is also suggested to influence the apparent amylose level or blue value (Zhao et al., 1998; Yamamori and Quynh, 2000), while genotypes or alleles affecting the amylopectin structure of wheat have not been studied in detail.

In rice, there are three alleles at the *Wx* locus, Wx^a, Wx^b and *wx*. A near-isogenic series introduced each of these alleles into the 'Taichung 65' genetic background was produced (Sano, 1984) and it has greatly contributed to genetic studies. Recently eight possible genotypes carrying different null *Wx* alleles of wheat have been developed (Zhao and Sharp, 1998; Miura et al., 1999; Yamamori and Quynh, 2000), but some variations ascribable to genetic backgrounds have been noticed (Miura et al., 1999; Yamamori and Quynh, 2000). Thus development of the partial and waxy mutants with a common genetic background will be much helpful in further accurate analyses of mechanisms of amylose synthesis and associated starch properties. The present study was conducted with the aims of producing eight possible genotypes of near-isogenic doubled haploids having different combinations of the three null alleles at the *Wx* loci, and characterizing their field performance and starch properties including amylose content, pasting property and chain-length distribution in amylopectin structure.

Materials and methods

Plant materials

Wheat F_1 hybrid seeds of the cross between the spring wheat cultivar, Chinese Spring (CS) and the waxy CS which were the initial genetic materials for this study were obtained in the 1997 spring season. CS possesses all three dominant alleles at the *Wx* loci responsible for the amylose synthesis and thus can produce all of the three Wx proteins. The waxy CS, deficient in all of the three Wx proteins, was selected from a three-way cross progeny among the single-chromosome substitution lines, CS*11/Kanto107 7A / CS*11/Kanto 107 4A // CS*9/Bai Huo 7D, and then successive backcrossings to CS (Miura et al., 1999).

Doubled haploid production

Doubled haploid plants were produced according to the method of Suenaga (1994) with modifications. Briefly, the F_1 plants grown in a growth chamber under the controlled temperature of 22°C were emasculated two to three days before anthesis and bagged. At stigma maturity, primary and secondary spikelets were pollinated with fresh pollen from the maize cultivar "Wase-homare".

Then 0.3 to 0.5 ml of 100ppm 2,4-D was injected to the base of upper most internode of the culm using a hypodermic syringe. Further, pollinated spikes were dripped with a few drops of 2,4-D and covered with translucent paper envelopes.

Fourteen days after pollination, embryos were rescued onto solidified half-strength Murashige and Skoog medium (pH 5.8). Cultured embryos were then incubated at 22° C in dark condition for two to three weeks and the germinated embryos were transplanted in culture tubes containing the same medium at 17° C with eight hours illumination. When the seedlings were at 1 to 1.5 months age with sufficient shoot and root development, they were transplanted on the vermiculate medium under the controlled temperature of 22° C in a growth chamber. Chromosome doubling was carried out in three to five tiller stages by treating plants with 0.07% colchicine solution. Selfed seeds from bagged heads were collected at maturity. In total 85 DH lines were produced in this experiment.

Identification and classification of DH lines

Half endosperms of each DH line were crushed and their starch granules were isolated according to the method described by Echt and Schwarts (1981). Then SDS-PAGE was performed for each DH line. Starch granule preparation and electrophoresis were performed as described by Nakamura et al. (1993) with the modification that a 15% SDS polyacrylamide gel with an acrylamide/BIS ratio of 30: 0.135 was used. The Wx proteins separated on electrophoretic gel were stained with silver stain kit (Wako Pure Chemical Industry Ltd, Japan). Classification of each DH line into the eight types (Table 1) was carried out at least three times. The remaining half endosperms including embryos were planted in pots for propagation.

Field experiment

Field trails were conducted at the experimental field of Obihiro University of Agriculture and Veterinary Medicine in the 1999 growing season. The 85 DH lines were sown in late April together with the parental CS and waxy CS as a single-row plot of 15 plants, spaced 10-cm within and 30-cm between rows. A randomized block design with two replicates was employed. Ear emergence time was scored as days to heading date from the 1st July. After anthesis, the experiment plots were covered to prevent pre-harvest sprouting. At maturity five random leading tillers were taken from each plot and used for evaluation of plant height, spikelet number/ear and 50-grain weight. The remainder of each plot was harvested for analyses of starch properties.

Amylose content

80 grams of grain samples of parental CS and each DH line harvested in the field experiment were conditioned to about 14% moisture content and were milled on a Barubender Qudramart Junior Test mill. Apparent amylose content per 100 mg of starch was colourimetrically estimated using

Autoanalyzer system II (Bran+Lubbe Co. Ltd) as described by Miura et al. (1994). The amylose content of Australian Standard White (ASW) was used as the standard. The assay was repeated at least three times.

Starch-pasting properties

Peak viscosity and breakdown of starch were measured using a Rapid Visco Analyzer (RVA, Newport Scientific Pvt. Ltd). Three grams of water-washed starch from flour were mixed with 25 ml of distilled water. The suspension was heated from 50° C to 95° C at the rate of 5° C /min and held at 95° C for 5 minutes, then cooled to 50° C at the rate of 5° C /min. The analysis was repeated at least twice. The breakdown was calculated as peak viscosity minus minimum viscosity. The parameters were given in Rapid Visco Units (RVU).

Analysis of amylopectin structure

The starch for the chain-length distribution analysis of amylopectin was isolated from two DH lines in each of the eight types of the Wx-protein deficient lines which were grown in a growth chamber where day and night temperatures were maintained at 22° C and 16° C, respectively. According to the method described by Nagamine and Komae (1996), high-performance anion exchange chromatography (HPAEC) was conducted using the DX-500 system (Dionex, CA, USA) equipped with a pulsed amperometric detecter (Model PED). The column used was a CarboPac PA-1 (250 x 4 mm I.D.) with a CarboPac PA-1 guard column (50 x 4 mm I.D.). For the HPAEC analysis, 50 μl of sample solution were injected using the autosampler and eluted at 1 ml/min with a linear gradient of 50 to 500 m*M* sodium acetate in 100 m*M* NaOH for 60 min.

The peak-area ratios (%) of degree of polymerization (DP) of 6 to 36 were calculated from elution profiles, and then the mol (%) of each DP was obtained. The unit-chains of DP were classified into three groups according to their periodic pattern described by Hanashiro et al. (1996) ; fa with DP 6-12, fb_1 with DP 13-24 and fb_2 with DP 25-36.

Results

Production and classification of DH lines

The presence and absence of different Wx proteins of 85 DH lines were investigated by SDS-PAGE. Eight electrophoretic phenotypes of the Wx proteins were found. Of the 85 DH lines, fourteen lines were included in type 1 as their banding pattern was coincide with that of the parental CS. 10, 8 and 12 single null lines were identified as types 2, 3 and 4 respectively, while 6, 11 and 10 lines were as double null types 5, 6 and 7 respectively. 11 DH lines did not produce any Wx proteins, so that they were identified as the complete waxy type. These waxy genotypes were confirmed to be amylose free by red-brown color when the cut-endosperm surface was stained with I_2/KI. The remaining

three DH lines showed novel banding patterns with a faint Wx-B1 or Wx-D1 protein. So these three lines were excluded in further analyses.

The eight electrophoretic phenotypes observed in the present experiment were corresponded to eight genotypes expected in the DH progeny derived from the plants heterozygous for all null alleles at each of the three *Wx* loci. If the null alleles are recessive when detected by the absence of the Wx proteins on electrophoretic gel separations, a segregation ratio of 1:1:1:1:1:1:1:1 in the doubled haploid population is hypothesized. The χ^2 analysis showed that the null alleles at each of the three *Wx* loci segregated in a Mendelian fashion (χ^2=0.405; 0.75<P<0.90) on both the pollen and egg sides.

Field performance

Analysis of variance for ear emergence time, plant height and grain yield traits indicated significant differences between DH lines but no differences among the eight types (data not shown). Table 2 summarized the results where no significant differences in the types were again confirmed by Tukey-Kramer method. Ear emergence time in the eight types ranged from 6.5 days (from July 1st) in type 8 to 9.6 days in the wild type. The types lacking the Wx-B1 protein tended to have earlier heading as found in types 3, 5, 7 and 8. Variation between the DH lines within types was large in the wild type and single null types. Plant height of CS was 119 cm. The eight types had around 120 cm of height. Similarly, both spikelets/ear and 50-grain weight in the eight types were identical to those of Chinese Spring.

Amylose content

CS produced 24.75% amylose (24.75 mg amylose per 100 mg starch granules). Amylose content in the eight types varied from 2.96% in type 8 to 24.58% in type 1 (Table 3). All single null types and double null types gave significantly lower amylose than that of the wild type. Even though the differences between single null types, 2, 3 and 4 could not be observed, a combination of null Wx-B1 and null Wx-D1 proteins in type 7 produced a significantly lower content that the other two double null types. These results demonstrated that for the capacity of amylose synthesis, the *Wx-A1a* allele is lowest and there was no significant difference between *Wx-B1a* and *Wx-D1a*. As shown by the standard deviation in Table 3, the line variation within the types was large in the single null types because these types included a few DH lines with lower amylose contents.

Starch-pasting properties

Association of lacks of any Wx protein with increases in starch-pasting properties was found in peak viscosity and breakdown (Table 3). Starch peak viscosity was highest in the waxy type, followed by three double null types, 5, 6 and 7. Three single null types were not different from the wild type.

Differences between single and double null types were clear for breakdown where the double null types and the waxy type gave significantly higher breakdown than the single null and the wild types. Although type 3 lacking the Wx-B1 protein tended to produce a higher breakdown, there were no statistical differences among three single null types and the wild type. Like peak viscosity the waxy type showed a highest breakdown that was almost twice of CS and single null types. Both parameters were negatively and significantly ($P<0.01$) correlated with amylose contents of the eight types ($r=-0.975$ for peak viscosity, $r=-0.971$ for breakdown).

Analysis of amylopectin structure

The HPAEC system used here can precisely separate malto-oligosaccharides according to their degree of polymelization under alkaline condition. The HPAEC profiles exhibiting the chain-length distribution of amylopectin fraction were apparently identical between the duplicate lines within the types and among the eight types of the Wx protein deficiency (Fig.1). When chains were classified into three groups, fa (DP6-12), fb_1 (DP13-24) and fb_2 (DP25-36), no obvious differences were again revealed in the analysis of variance (Table 4). These results indicated that no segregation of genes affecting amylopectin structure in the genetic background and the reduced GBSS I activity due to introgression of the null *Wx* alleles does not affect both the chain length and the degree of branching of amylopectin.

Discussion

The number of DH lines classified into the eight genotypes of the Wx protein deficiency fitted to the theoretical ratio of an equal frequency. This confirms the result of Zhao et al. (1998) who practiced the production of the eight genotypes through wheat × maize method for the first time and showed that the null alleles at each of three *Wx* loci segregate according to a Mendalian fashion. In the present study using near-isogenic parents, CS and the waxy CS in the initial cross, it was further indicated that the chromosome segments locating the *Wx* loci do not carry genes involving distorted segregation. A random assortment of parental alleles from heterozygous F_1 plants to fixed populations like DH lines is desirable in breeding and genetics. Therefore, it would easily permit to utilize these null *Wx* alleles in conventional breeding programs for improving starch quality.

At the setting up this study, we took interest in if a reduced amylose content due to introgression of the null *Wx* alleles brings about a reduction in grain size or grain yield. The field experiment indicated that variation for grain yield traits were small and statistically undetectable among the eight types. This implies that wheat breeders can manipulate any *Wx* loci with less attention to a yield reduction. Only for ear emergence time, the types lacking the Wx-B1 protein tended to be earlier than the wild type as found in types 3, 5, 7 and 8 (Table 2). A QTL, designated *QEet.ocs-4A.1*, with a large effect on heading time was identified at, or closely linked, with the

Wx-B1 locus on chromosome 4A (Araki et al., 1999). Since associated with the null *Wx-B1b* is an effect on earlier heading, probably accelerated heading in the four types lacking the Wx-B1 protein in common is due to a tight linkage of *QEet.ocs-1* with *Wx-B1*. Such a close linkage between the *Wx* locus and a heading date QTL, *Hd-3* is also found on rice chromosome 6 (Dung et al., 1998).

The results of amylose content indicated that the amylose synthesis capacity of *Wx-A1a* allele is the lowest but that of *Wx-B1a* and *Wx-D1a* is not distinguishable because there was no significant difference in the amylose contents of type 5 and type 6 (Table 3). These confirm our previous results (Miura and Sugawara, 1996; Miura et al., 1999). Furthermore, we detected a linear relationship of amylose contents in the eight types with peak viscosity and/or breakdown. This is ascribable to the differential effects of the null alleles at the three *Wx* loci on both amylose content and starch-pasting properties. With respect to the ability of reduction in amylose and increase in starch-pasting properties, the null *Wx-B1b* is mostly responsible for, followed by *Wx-D1b* with little effect of *Wx-A1b* (Araki et al., 2000; Yamamori and Quynh, 2000). This evidence would explain in most parts of a negative correlation of amylose content with peak viscosity observed in the cultivar level (Moss, 1980; Oda et al., 1980; Zeng et al., 1997). However, the molecular mechanisms causing differential effects of three *Wx* genes on amylose content and pasting properties are not fully understood.

In cultivated rice, there are two wild type alleles, Wx^a and Wx^b. Wx^a is characteristic of Indica rice and Wx^b is found mainly in Japonica rice. Consistent with the high levels (about 10-hold) of the *Wx* transcript or Wx protein in Wx^a, amylose content in Indica type is higher than that in Japonica type (Sano, 1984). While there is only one amino acid difference between Wx^a and Wx^b gene products, it is likely that their specific activities are similar (Okagaki, 1992). To explain this conflict, it has been pointed out that variation in the amounts of the Wx protein and amylose content in Japonica and Indica types is regulated at the level of Wx transcript processing and, more specifically, at the stage of intron I excision from the Wx pre-mRNA (Okagaki, 1992; Wang et al., 1995). Molecular analyses suggest that GT sequence at the 5' splice junction of intron I is essential for the high expression level in Wx^a which can produce a larger amount of the Wx protein (Cai et al., 1998; Hirano et al., 1998; Isshiki et al., 1998). On the other hand, a GT to TT mutation in Wx^b produces abnormal splicing of the first intron, resulting in a less amount of Wx protein and a lower content of amylose in Japonica type. This is supported by the relation between the polymorphism at 5' splice junction and amylose content by analyzing 92 U.S. rice cultivars (Ayres et al., 1997). In wheat, the *Wx* gene dosage is linearly related to the amounts of the Wx proteins but not to amylose content (Miura et al., 1999; Yamamori and Quynh, 2000). Whether the efficiency of excision of introns in each *Wx* gene associated with differences in amylose content is not determined. So these subjects are potential areas for further research using the near-isogenic DH lines of the Wx protein deficiency.

It has been reported that there is a difference in amylopectin structure between the wild type and its waxy mutant of *Chlamydomonas* (Delrue et al., 1992). In wheat, there are little information available for the relationship between the GBSS I activity and amylopectin structure. Yasui et al. (1996) found in a chain-length distribution analysis that amylopectin of the waxy wheat is structurally identical to that of their non-waxy parents. Zhao et al. (1998) also detected no difference between the *Wx-B1a* and *Wx-B1b* lines in the percentage of chains with a degree of polymerization of more than 40 glucose residues. In these reports, however, a possibility of the segregation of genes affecting amylopectin structure or starch properties was not taken into account. To our knowledge, the deficiency of starch granule proteins of 100- to 105-kDa (SPG-1) derives some alterations in structure of amylopectin with apparent high amylose (Yamamori et al., 2000). The SGP-1 null genotype has increased fa (DP6-12) chains and reduced fb_1 (DP13-24) chains. Probably fa chains and fb_1 chains correspond to A and B1 chains of amylopectin, respectively (Hanashiro et al., 1996). Our eight types of DH lines showed no alterations in fa and fb_1 chain groups (Table 4, Fig.1), irrespective of great changes of the amylose/amylopectin ration. This suggests that the reduced GBSS I activity does not influence the SGP-1 activity. To analyze the interaction between GBSS I activity and the SGP-1 deficiency, genetic materials introduced the SGP-1 null alleles into the near-isogenic DH lines of the Wx protein deficiency would be desirable.

References

Araki E, Miura H, Sawada S (1999) Identification of genetic loci affecting amylose content and agronomic traits on chromosome 4A of wheat. Theor Appl Genet 98:977-984.

Araki E, Miura H, Sawada S (2000) Differential effects of the null alleles at the three *Wx* loci on the starch pasting properties of wheat. Theor Appl Genet 100:113-1120.

Ayres NM, McClung AM, Larkin PD, Bligh HFJ, Jones CA, Park WD (1997) Microsatellites and a single-nucleotide polymorphism differentiate apparent amylose classes in an extended pedigree of US rice germplasm. Theor Appl Genet 94: 773-781.

Cai XL, Z.Y.Wang ZY, Xing YY, Zhang JL, Hong MM (1998) Aberrant splicing of intron 1 leads to the heterogeneous 5' UTR and decreased expression of *waxy* gene in rice cultivars of intermediate amylose content. Plant J 14:459-465.

Chao S, Sharp PJ, Worland AJ, Warham EJ, Koebner RMD, Gale MD (1989) RFLP-based genetic maps of wheat homoeologous group-7 chromosomes. Theor Appl Genet 78: 495-504.

Delrue B, Fontaine T, Routier F, Decq A, Wieruszeski J-M, Van Den Koornhuyse N, Maddelein M-L, Fournet B, Ball S (1992) Waxy *Chlamydomonas reinhardtii*: monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. J Bacteriol 174: 3612-3620.

Dung LV, Inukai T, Sano Y (1998) Dissection of a major QTL for photoperiod sensitivity in rice: its

association with a gene expressed in an age-dependent manner. Theor Appl Genet 97: 714-720.

Echt C, Schwartz D (1981) Evidence for the inclusion of controlling elements within the structural gene at the *waxy* locus in maize. Genetics 99: 275-284.

Hanashiro I, Abe J, Hizukuri S (1996) A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. Carbohydr Res 283: 151-159.

Hirano H-Y, Eiguchi M, Sano Y (1998) A single base change altered the regulation of the Waxy gene at the posttranscriptional level during the domestication of rice. Mol Biol Evol 15: 978-987.

Isshiki M, Morino K, Nakajima M, Okagaki RJ, Wessler SR, Izawa T, Shimamoto K (1998) A naturally occurring functional allele of the rice *waxy* locus has a GT to TT mutation at the 5' splice site of the first intron. Plant J 15:133-138.

Miura H, Sugawara A (1996) Dosage effects of the three *Wx* genes on amylose synthesis in wheat endosperm. Theor Appl Genet 93:1066-1070.

Miura H, Tanii S, Nakamura T, Watanabe N (1994) Genetic control of amylose content in wheat endosperm starch and differential effects of three *Wx* genes. Theor Appl Genet 89: 276-280.

Miura H, Araki E, Tarui S (1999) Amylose synthesis capacity of the three *Wx* genes of wheat cv. Chinese Spring. Euphytica 108: 91-95.

Moss HJ (1980) The pasting properties of some wheat starches free of sprout damage. Cereal Res Comm 8:297-302.

Nagamine T, Komae K (1996) Improvement of a method for chain-length distribution analysis of wheat amylopectin. J Chromatography A 732:255-259.

Nakamura T, Yamamori M, Hirano H, Hidaka S (1993) Identification of three waxy proteins in wheat (*Triticum aesitivum* L.). Biochem Genet 31: 75-86.

Nakamura T, Yamamori M, Hirano H, Hidaka S, Nagamine T (1995) Production of waxy (amylose free) wheats. Mol Gen Genet 248: 253-259.

Oda M, Yasuda Y, Okazaki S, Yamauchi Y, Yokoyama Y (1980) A method of flour quality assessment for Japanese noodles. Cereal Chem 57:253-254.

Okagaki RJ (1992) Nucleotide sequence of a long cDNA from rice *waxy* gene. Plant Mol Biol 19: 513-516.

Sano Y (1984) Differential regulation of *waxy* gene expression in rice endosperm. Theor Appl Genet 68: 467-473.

Suenaga K (1994) Doubled haploid system using the intergenetic cross between wheat (*Triticum aestivum*) and maize (*Zea mays*). Bull Natl Inst Agrobiol Resour 9: 83-139.

Toyokawa H, Rubenthaler GL, Powers JR, Schanus EG (1989) Japanese noodle qualities. I. Flour components. Cereal Chem 66: 382-386.

Wang ZY, Zheng FQ, Shen GZ, Gao JP, Snustad DP, Li MG, Zhang JL, Hong MM (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy*

gene. Plant J 7: 613-622.

Yamamori M, Quyhn NT (2000) Differential effects of Wx-A1, -B1 and -D1 protein deficiencies on apparent amylose content and starch pasting properties in common wheat. Theor Appl Genet 100:32-38.

Yamamori M, Fujita S, Hayakawa K, Matsuki J, Yasui T (2000) Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. Theor Appl Genet 101: 21-29.

Yasui T, Matsuki J, Sasaki T, Yamamori M (1996) Amylose and lipid contents, amylopectin structure, and gelatinisation properties of waxy wheat (*Triticum aestivum*) starch. J Cereal Sci 24: 131-137.

Zeng M, Morris CF, Batey IL, Wrigley CW (1997) Sources of variation for starch gelatinization, pasting and gelation properties in wheat. Cereal Chem 74: 63-71.

Zhao XC, Sharp PJ (1998) Production of all eight genotypes of null alleles at 'waxy' loci in bread wheat, *Triticum aestivum* L. Plant Breed 117: 488-490.

Zhao XC, Batey IL, Sharp PJ, Crosbie G, Barclay I, Wilson R, Morell MK, Appels R (1998) A single genetic locus associated with starch granule properties and noodle quality in wheat. J Cereal Sci 27: 7-13.

Table 1. The frequency of the eight Wx phenotypes in the doubled haploid progeny derived from the F_1 plants between Chinese Spring and the waxy CS

	Wx proteins present			Number of doubled haploid progeny	
Type	Wx-A1	Wx-B1	Wx-D1	Expected	Observed
CS	+	+	+		
1. wild type	+	+	+	10.25	14
2. wxA	–	+	+	10.25	10
3. wxB	+	–	+	10.25	8
4. wxD	+	+	–	10.25	12
5. wxAB	–	–	+	10.25	6
6. wxAD	–	+	–	10.25	11
7. wxBD	+	–	–	10.25	10
8. wxABD	–	–	–	10.25	11

Table 2. Ear emergence time, plant height and grain yield traits of the eight types of the Wx-protein deficiency

Type	Ear emergence time (days from 1st ,July)	Plant height (cm)	Spikelets/ear	50-grain weight (gram)
CS	9.0	119.0	15.4	1.714
1 wild type	9.6±5.1a[1)]	116.4±6.0a	15.3±0.69a	1.658±0.106a
2 wxA	9.1±4.2a	120.9±4.9a	15.4±1.05a	1.707±0.073a
3 wxB	7.7±2.2a	115.3±6.2a	15.0±1.03a	1.696±0.077a
4 wxD	8.7±3.9a	117.6±7.8a	15.7±0.86a	1.718±0.078a
5 wxAB	6.7±1.5a	123.8±4.7a	14.7±0.54a	1.730±0.097a
6 wxAD	8.2±1.6a	119.6±7.2a	15.3±1.12a	1.730±0.053a
7 wxBD	7.3±1.4a	117.7±6.0a	14.9±1.25a	1.746±0.088a
8 wxABD	6.5±0.8a	123.1±5.5a	14.7±0.89a	1.704±0.053a

1) Mean performances ± variation between lines (SD). Means followed by the same letter in the same column are not significantly different at $p = 0.05$

Table 3. Amylose content and starch-pasting properties in the eight types of the Wx-protein deficiency

Types	Amylose content (%)	Starch-pasting properties	
		Peak viscosity (RVU)	Breakdown (RVU)
CS	24.75	220.5	104.0
1. wild type	24.58±0.60 a[1)]	216.3±29.6 ab	90.6±20.5 a
2. wxA	22.63±1.63 b	209.7±15.8 a	98.9±16.5 a
3. wxB	22.18±1.15 b	228.2±12.6 abc	116.7±13.9 ab
4. wxD	22.86±1.16 b	225.7±12.4 abc	106.6±13.4 a
5. wxAB	18.57±0.39 c	236.9±6.7 bc	141.7±11.1 bc
6. wxAD	18.75±0.82 c	240.0±18.3 c	141.0±19.8 bc
7. wxBD	16.24±0.65 d	243.1±12.3 c	145.63±10.5 c
8. wxABD	2.96±0.40 e	291.5±35.7 d	203.8±16.9 d

1) Mean performances ± variation between lines (SD). Means followed by the same letter in the same column are not significantly different at $p = 0.05$

Table 4. The distribution of unit-chain groups in the duplicate lines of the eight types of the Wx protein deficiency

Types	DH lines	Unit-chain groups (mol %)		
		fa(DP6-12)[a)]	fb_1(DP13-24)	fb_2(DP25<)
1	CS	48.0	42.9	9.1
	DH29	47.1	44.3	8.6
2	DH7	47.4	43.8	8.8
	DH8	48.1	43.9	8.0
3	DH19	47.0	44.6	8.4
	DH22	46.2	45.3	8.5
4	DH21	46.8	44.5	8.7
	DH24	46.1	45.2	8.7
5	DH5	46.7	44.5	8.9
	DH44	47.2	44.0	8.9
6	DH3	47.2	44.1	8.7
	DH14	47.3	44.8	7.9
7	DH1	47.9	43.4	8.7
	DH9	47.9	43.7	8.4
8	DH37	50.1	42.0	7.9
	DH51	48.3	43.2	8.5
LSD (5% level)		1.7	1.5	0.8

a) Degree of polymelization, see text.

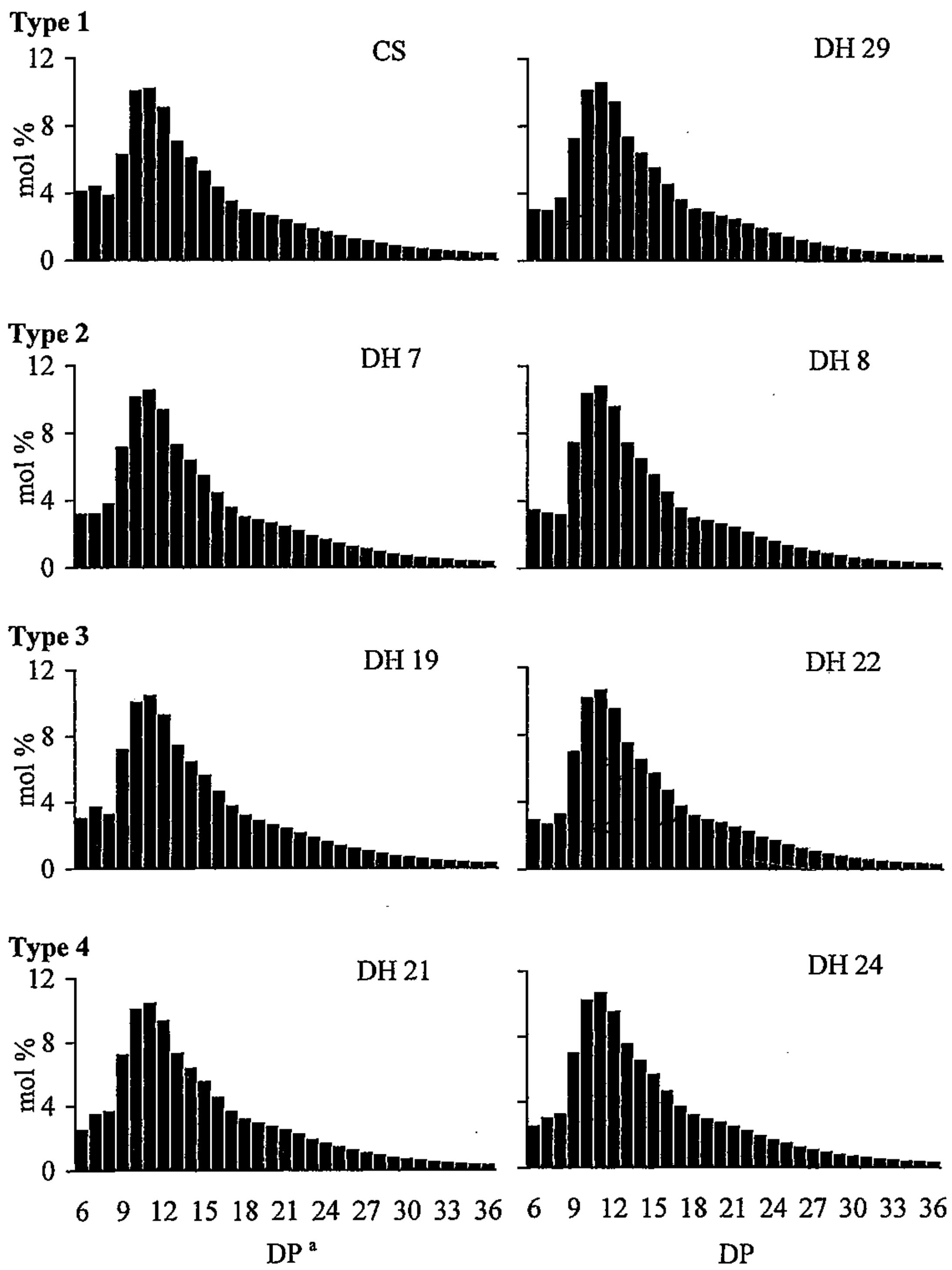
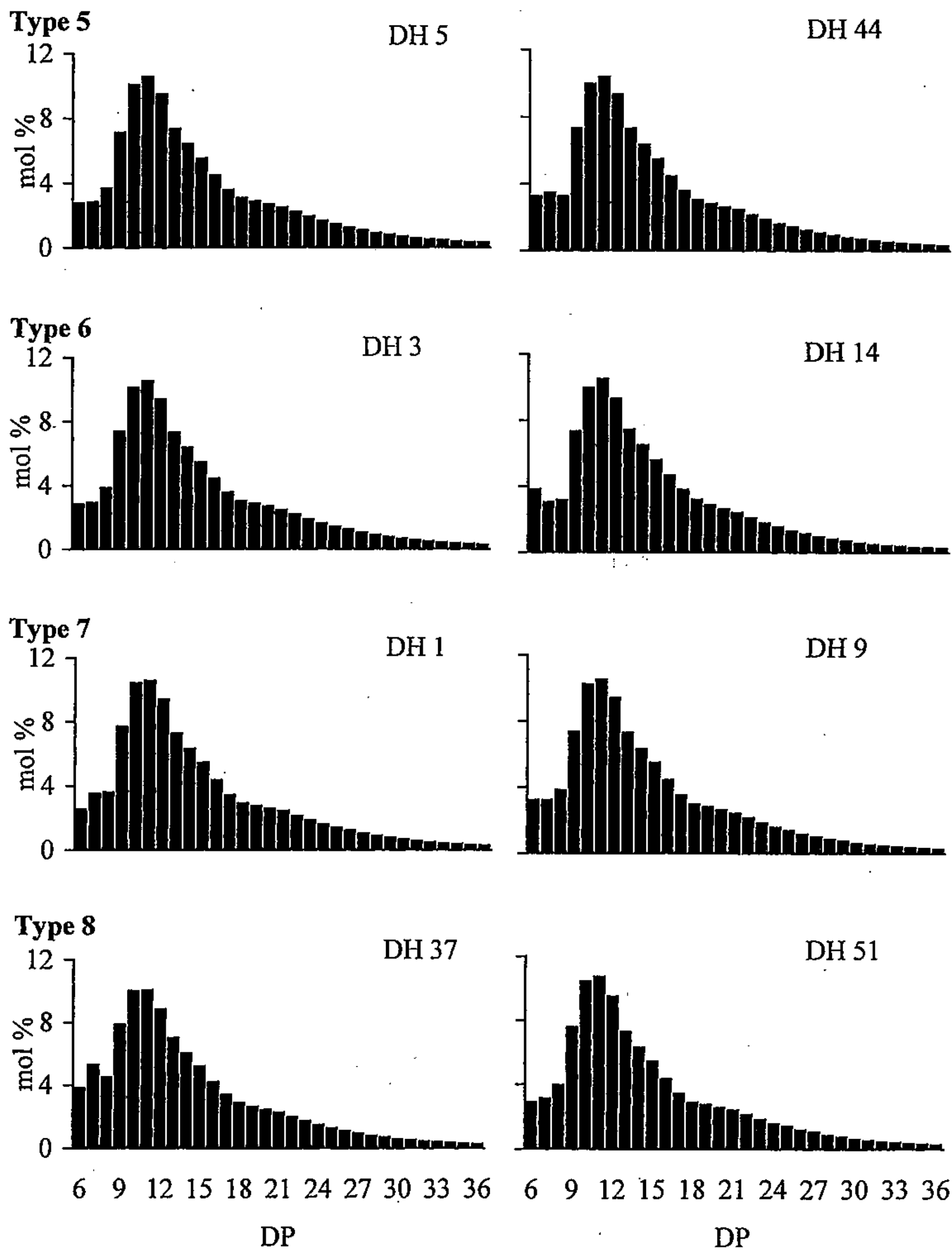


Fig. 1. The chain-length distribution profiles of the debranched starch extracted from the near-isogenic eight genotypes of the Wx-protein deficiency.


Type 5
DH 5
DH 44
Type 6
DH 3
DH 14
Type 7
DH 1
DH 9
Type 8
DH 37
DH 51
mol %
12
8
4
0
6 9 12 15 18 21 24 27 30 33 36
DP

Chapter 4. Identification of genetic loci affecting amylose content and agronomic traits on chromosome 4A

Abstract

Chromosome 4A of wheat carries the *Wx-B1* gene encoding granule-bound starch synthase involved in amylose synthesis in the endosperm. To determine the pleiotropic effects of this locus and effects of independent QTLs on agronomic traits, genetical analysis of chromosome 4A was conducted using 98 single chromosome recombinant substitution lines derived from a cross of Chinese Spring and Chinese Spring *11/Kanto107 4A with a low amylose content due to the null *Wx-B1b* allele. For amylose content, the most part of the genetic variation was explained by the allelic difference at the *Wx-B1* locus. An additional QTL of a minor effect was mapped in the 6.2 cM *Xbcd1738/Xcdo1387* interval on the short arm, where the allele from Kanto107 increased amylose content. Field trials over two seasons revealed a pleiotropic effect of *Wx-B1* or the effect of a closely linked QTL on ear emergence time. A QTL linked to *Wx-B1* was detected for plant height. For plant yield and its components, there was no evidence for significant main effects associated with *Wx-B1* or adjacent regions. One plant yield QTL was identified by RFLP markers on the short arm and this was identical to QTLs controlling spikelet number/ear and grain weight/ear. At these QTLs for agronomic traits, alleles from Kanto107 contributed to an earlier emergence time, height reduction and yield increase.

Key words ; *Triticum aestivum* L., amylose content, QTLs, *Wx-B1*, yield traits

Introduction

Starch is the major component of the wheat grain, making up 65-70% of the dry weight of mature grain (Rahman et al., 1995). Starch consists essentially of two different forms of polymers of glucose, linear amylose and branched amylopectin. The amylose/amylopectin ratio of starch is extremely important in producing marketable flour products, because it affects quality of end-uses (Moss, 1979; Oda et al., 1980; Toyokawa et al., 1989). Low-amylose-content cultivars are preferred for manufacture of certain noodle types (Yamamori et al., 1992; Miura and Tanii, 1994) and the potential use of starch with reduced amylose content is a current topic of discussion among wheat breeders and geneticists. Therefore, a better understanding of the factors modifying amylose content is required for the development of effective selection or screening procedures for use in breeding.

The endosperm contains two distinct types of starch synthases, granule-bound starch synthase (GBSS), and soluble starch synthases which act together with starch-branching enzyme to synthesize amylopectin. The major GBSS I of 60 kDa, so called the Wx protein, is the key enzyme

involved in amylose synthesis, and is encoded by the *Wx* loci (Tsai, 1974; Echt and Schwartz, 1981).

In hexaploid wheat (*Triticum aestivum* L.), three Wx proteins, Wx-A1, Wx-B1 and Wx-D1, are coded by the three homoeologous *Wx* loci, *Wx-A1*(7AS), *Wx-B1*(4AL) and *Wx-D1*(7DS), respectively (Chao et al., 1989; Nakamura et al., 1993a). The three *Wx* genes have different effects on amylose content, in particular the null *Wx-B1b* allele is associated with the largest reduction through the lack of the Wx-B1 protein (Miura et al., 1994; Miura and Sugawara, 1996). Cultivars that are accepted into white salted noodle market classifications lack the Wx-B1 protein (Miura and Tanii, 1994; Yamamori et al., 1994; Zhao et al., 1998). In breeding programs aimed to improve noodle quality, the genetic manipulation of *Wx-B1* and chromosome 4A seems to be a prerequisite. However, whether the *Wx-B1* locus and the adjacent region of 4AL affect agronomically and economically important traits, such as flowering time and plant yield, has not been reported.

A low-amylose content of the Japanese spring wheat 'Kanto107' has received much attention in breeding programs. This cultivar lacks both of the Wx-A1 and Wx-B1 proteins and only the Wx-D1 protein produced by *Wx-D1a* synthesizes amylose (Nakamura et al., 1993b). Genetic variation associated with chromosome 4A for important agronomic traits was noted, when the null allele at the *Wx-B1* loci of 'Kanto107' was introduced into a 'Chinese Spring' (CS) genetic background by producing the single-chromosome substitution line (Miura and Sugawara, 1996). In this paper, we report the effects of allelic difference at the *Wx-B1* locus on amylose content and quantitative agronomic traits, together with effects of independent quantitative trait loci (QTLs).

Materials and methods

Plant material

The CS*11/Kanto107 4A substitution line had previously been developed by eleven backcrosses of the monosomic substitution to CS monosomic 4A prior to the extraction of the disomic substitution. CS*11/Kanto107 4A had been shown to lack the Wx-B1 protein, with a null *Wx-B1b* allele, which has a 2-3% lower amylose content than CS (Miura and Sugawara, 1996).

Using the procedures described by Law (1966), homozygous recombinant substitution lines were developed for chromosome 4A from the F_1 between CS and CS*11/Kanto107 4A. In total 98 different, homozygous disomic plants were extracted, then grown to maturity and allowed to self-pollinate.

Identification of the *Wx-B1* allele type and measurement of amylose content

To classify the recombinant substitution lines for the *Wx-B1* allele, electrophoretic analysis of starch granule bound protein were performed. Starch granule preparation and SDS-PAGE were conducted as described by Nakamura et al. (1993a), with the modification that a 15% SDS polyacrylamide gel

with an acrylamide/BIS concentration of 30:0.135 was used for electrophoresis.

For evaluation of amylose content in the endosperm, all recombinant lines and the parents were grown in the field in 1996 and 1997. After anthesis, all lines were covered to prevent pre-harvest sprouting. Grain samples were milled on a Brabender Quadrant Junior Test Mill to a final extraction rate of a 60%. Starch granules were separated using conventional methods. The amylose content per 100 mg of starch granules was colorimetrically determined using the Auto Analyzer System II (Bran+Lubbe Co.) as described by Miura et al.(1994).

RFLP analysis

DNA of each recombinant line and parent was extracted from young leaves by a modified CTAB method (Murray and Thompson, 1980). Restriction enzyme digestion, southern blotting, and hybridization were performed as described in Kato et al.(1998). PSR clone libraries, wheat cDNA or genomic DNA, were obtained from Dr. MD Gale, John Innes Centre, UK. BCD clone libraries, barley cDNAs, and CDO clone libraries, oat cDNAs, were obtained from Dr. ME Sorrells, Cornell University, USA. Of these, the probes which were known from previous reports (Gale et al., 1995; Nelson et al., 1995) to be located on chromosome 4A were utilized. Using a "Gene Images" kit (Amersham LIFE SCIENCE), a non-isotope labeling and detecting system, the 98 recombinant lines were genotyped for RFLP markers polymorphic between the parents.

Chi-square analysis was performed between the observed segregation ratio of each RFLP marker and the theoretical 1:1 ratio. The linkage map was constructed using MAPMAKER/ EXP3 (Lander et al., 1987), and the recombination frequencies were converted to centiMorgans (cM) using the Kosambi mapping function (Kosambi, 1944). The marker ordering, distance and degree of chromosome coverage were assessed via comparisons with the consensus maps of Gale et al. (1995) and Nelson et al. (1995).

Field trials

Field trials were conducted at the experimental field of Obihiro University of Agriculture and Veterinary Medicine over two seasons, 1996 and 1997. The parental lines and the recombinant lines were grown as a single row plot of 12 plants, spaced 10 cm within and 30 cm between rows. A randomized block design with five replicates was used. Ear emergence time was scored as days to heading date from the 1st July. At maturity five random leading tillers were taken from each plot and used for evaluation of plant height and yield components, including spikelet number/ear, grain weight/ear and 50 grain weight. The remainder of each plot was harvested to score plant yield and tiller number/plant.

Detection of QTL

Analyses of variance (ANOVA) were used to detect differences between parents and to partition variation between recombinant lines, between seasons and line x season interaction. Estimates of variance components, including genetic variance and genotype by environment interaction variance, were calculated by equating the mean squares to their expected values. They were used to estimate heritability values. A one-way ANOVA was used for each marker to detect significant differences between allele class means by comparing it with the variation between lines within classes.

QTL analysis was also performed using the software package MQTL (Tinker and Mather 1995) which can detect both QTL main effects and QTL x season interaction. The phenotype data sets were analyzed by the simple interval mapping (SIM) and simplified composite interval mapping (sCIM) procedures. The linkage group was scanned at a 5 cM interval test statistic. Six even-spaced background markers were specified for sCIM. Except for multiple-environment sCIM analysis, where precise test statistics are not computable, type-I 5% significance thresholds were established with 1000 permutations. QTL inference were made as QTLs were declared at positions where SIM peaks were significant for either QTL main effects or QTL x season interaction with strong sCIM peaks.

Results

Linkage map

Of the 98 recombinant lines, 47 lines were found to produce the Wx-B1 protein by the SDS-PAGE system, thus identified as the CS type with *Wx-B1a*. The remaining 51 lines were deficient in the Wx-B1 protein and identified as the CS*11/Kanto107 4A type with *Wx-B1b*. CS chromosome 4A carries a dominant allele at the awn inhibitor *Hd* locus (Sears, 1954) and suppresses awn development, whereas CS(Kanto107 4A) confers an awned phenotype by the recessive *hd* allele. The recombinant substitution lines were classified by eye into unawned, 51 lines, with *Hd*, and awned, 47 lines, with *hd*.

Of RFLP markers detecting polymorphism between the parents, eight markers showing non-distorted segregation from the expected 1:1 ratio were used for map construction. The resulting genetic linkage map is illustrated in Fig.1. Compared to previously reported maps by Gale et al. (1995) and Nelson et al. (1995), the map covered approximately 107 cM, from the terminal region of the long arm defined by *Xpsr160* to the central part of the short arm as marked by *Xpsr163*. The *Wx-B1* mapped to the long arm at about 60 cM distance from the centromere, and closely linked to *Xpsr115*, a proximal marker to the 7BS/5AL breaking point.

Amylose content

Starch granules from CS produced about 25.0% amylose content over two seasons. CS*11/Kanto107 4A showed a consistently, and significantly, lower content by about 2.0% than CS.

The amylose content in the recombinant lines is given in Fig.2. The 22.1 - 25.1% range in the recombinant lines was consistent between seasons and a highly significant correlation was detected (r=0.75, P<0.001) with a heritability value of 0.696. As expected, the deficiency of the Wx-B1 protein due to the null *Wx-B1b* allele caused a clear reduction in the amylose content. MQTL analysis showed that the allelic difference at *Wx-B1* was a major factor accounting for more than 70% of the variation in each season (Fig.1a).

However, the 51 lines with the null *Wx-B1b* had a distribution from 22.1 to 23.9% amylose with a significant correlation between seasons (r=0.37, p<0.01) and the remaining 47 lines with *Wx-B1a* varied from 23.1 to 25.1%, suggesting the involvement of an additional genetic factor(s). To determine if the variation of about 2.0% observed between lines within the allele classes included genetic variation, QTL analysis was carried out separately for the *Wx-B1a* and *Wx-B1b* classes (Fig.1b,c). In the *Wx-B1b* allele class, both ANOVA and MQTL revealed a significant effect associated with the *Xbcd1738/Xcdo1387* interval on the short arm. This QTL, designated *QAmc.ocs-4A.1*, accounted for about 17% of the variation in the lines within the *Wx-B1b* class. The Kanto107 allele at *QAmc.ocs.1* produced a higher amylose content with an additive effect of 0.3%. In the *Wx-A1a* allele class, ANOVA confirmed the minor effect associated with *Xcdo1387*, whilst MQTL analysis failed to detect this effect.

Analysis of agronomic traits

The mean phenotypes of the parents, mean and range in the recombinant lines for the agronomic traits are presented in Table 1. ANOVA detected highly significant differences (p<0.001) between the recombinant lines for each trait. The estimated heritability values ranged from 0.216 for tiller number/plant to 0.698 for spikelet number/ear. These suggest that chromosome 4A has effects on the traits examined and that these genetic factors segregate among the recombinant lines. Line x season interactions were also significant for all traits except spikelet number/ear. However, when the expected variance components were extracted from mean squares of ANOVA tables, it was found that line x season interactions contributed around 6% or less of the total variation (2.80% for grain weight/ear - 6.50% for tiller number/plant).

Ear emergence time and plant height

Over two seasons of trials, CS*11/Kanto107 4A flowered 2.6 - 3.0 days earlier than CS (Table 1). One QTL with a large effect was identified at, or closely linked with the *Wx-B1* locus (Fig. 3). ANOVA showed that the effect was most strongly associated with *Wx-B1*. The allele from CS*11/Kanto107 4A contributed to accelerated flowering, having additive effects of 1.2 days (1996) and 1.6 days (1997). This QTL designated *QEet.ocs-4A.1* accounted for approximately 40% of variation in the lines in each season (Table 2).

The mean height of CS was 109.2 cm (1996) and 114.4 cm (1997), approximately 8 - 9 cm higher than CS*11/Kanto107 4A. The recombinant lines ranged from 91.5 cm to 110.7 cm in the 1996 trial, and from 95.9 cm to 120.1 cm in the 1997 trial. However, the lines having heights significantly greater than the CS and shorter than CS*11/Kanto107 4A were not observed, showing the absence of transgressive segregation. A QTL, *QHt.ocs-4A.1*, was detected in the 21.7 cM *Xpsr119/ Wx-B1* interval (Fig. 3), close to the latter locus where the height-reducing allele came from CS*11/Kanto107 4A with additive effects of 4.8 cm (1996) and 5.7 cm (1997). This primary QTL accounted for about 30% of the line variation. The second QTL, *QHt.ocs-4A.2* of lesser effect was identified in the *Xbcd1738/Hd* interval on the short arm, which explained 20% (1996) and 26% (1997) of the line variation. Like *QHt.ocs-4A.1*, the Kanto107 allele at this QTL produced a reduced height.

Plant yield and its components

While the two parents did not differ widely in plant yield, the recombinant progeny showed a large variation, ranging from 4.7 g to 12.8 g (1996) and from 7.4 g to 17.6 g (1997). Plant yield in the 1996 trial was about two-thirds of that in the 1997 trial, being partly due to head scab (*Microdochium nivale* Samuels et Hallett) infection. Compared to the parents, the recombinant lines contained several lower-yielding lines. Over the two seasons, 14 lines showed significantly lower yield performance than the parents, indicating the occurrence of transgressive segregation.

As shown in Fig. 4, there was no evidence for significant main effects of the *Wx-B1* locus or its adjacent regions on plant yield. One QTL, designed *QYld.ocs-4A.1* was detected by *Xbcd1738* on the short arm. At *QYld.ocs-4A.1*, the yield increasing allele came from the CS*11/Kanto107 4A with the additive effects of 1.4 g (1996) and 1.7 g (1997) which explained 27% and 20% of the variability, respectively (Table 2).

Of yield components, a QTL for spikelet number/ear, *QSpn.ocs-4A.1*, and that for grain weight/ear, *QGwe.ocs.-4A.1*, were highly genetically correlated with each other and mapped to very similar positions to *QYld.ocs-4A.1* (Fig. 4). *QSpn.ocs-4A.1* explained approximately 50% of total phenotypic variance in the recombinant lines, and the Kanto107 allele with additive effects of 1.8 (1996) and 1.4 (1997) contributed to increased spikelet number. The high grain weight/ear allele at *QGwe.ocs-4A.1* again came from CS*11/Kanto107 4A, and had additive effects of 0.14 g (1996) and 0.09 g (1997) accounting for 27% and 12% of the phenotypic variation, respectively (Table 2). MQTL detected QTL x season interactions on the long arm for spikelet number/ear and grain weight/ear, suggesting the minor effects but these were much smaller than the main effects of *QSpn.ocs-4A.1* and *QGwe.ocs-4A.1*. Most of the lower-yielding segregants also produced lower spikelet number and grain weight/ear than the lower parent.

On the other hand, one QTL associated with tiller number/plant, *QTn.ocs-4A.1*, was found at

the most distal marker *Xpsr163* of the short arm (Fig. 4). The additive effects of this QTL were small and accounted for only 10% (1996) and 16% (1997) of the phenotypic variance (Table 2). In the 1996 trial, a QTL for 50 grain weight, *QFgw.ocs-4A.1*, was detected in the centromere region flanked with *Xbcd265* and *Xbcd1738*.

Discussion

The recombinant substitution analysis of this study confirms a close association of a reduction in amylose content with lack of the Wx-B1 protein encoded by the null *Wx-B1b* allele. Reduced-amylose wheats have been shown to confer superior performance in some noodle applications and may confer enhanced water absorption to baked goods. The null *Wx-B1b* is also suggested to contribute to improvement of starch paste properties such as a high peak viscosity (Zhao et al., 1998; Araki et al., 2000) which is an important factor for noodle (and bread) manufacture. Compared to single null lines like CS*11/Kanto107 4A or double null lines with *Wx-A1b* and *Wx-D1b*, double null lines with *Wx-B1b* and *Wx-D1b* or with *Wx-B1b* and *Wx-A1b* are more likely to provide consistent sources of low amylose starch and high paste viscosity (Miura et al., 1998). Therefore, it is preferred that double null cultivars and advanced breeding lines should be used to carry *Wx-B1b* into the development of locally adapted wheats carrying desirable starch properties.

Even though amylose content is mostly influenced by the *Wx* genes, we show that they do not explain all variation in this trait. A modifying factor, *QAmc.ocs-4A.1* was detected in the present study and mapped in the 6.2 cM *Xbcd1738/Xcdo1387* interval on the short arm. This QTL might account for variation within the *Wx-B1* allele classes. Since the effect of *QAmc.ocs-4A.1* was masked in lines with *Wx-B1a*, an epistatic interaction with *Wx-B1* appeared to decrease the action of *QAmc.ocs-4A.1* in the presence of the Wx-B1 protein. The availability of genetic loci which alter amylose content around 0.5 to 1.0% make it feasible to "fine-tune" genotypes to meet specific requirements of flours. Molecular markers will allow the *QAmc.ocs-4A.1* locus with minor effect to be used more precisely to manipulate amylose content than would be possible on the basis of phenotypic selection.

For changing amylose content, at least two kinds of genes should be considered ; one associated with starch synthesis and another with starch hydrolysis. As far as is known, chromosome 4A carries no loci encoding other proteins, except Wx-B1, mediating soluble starch synthases and starch branching enzyme. The short arms of the group 7 chromosomes has homoeologous genes encoding starch granule-bound proteins with molecular weights of 100-105 kDa which have starch-synthase activity (Denyer et al., 1995). The possibility that the translocated segment on 4A from 7BS contains the gene is refuted by the fact that 7BS produces the starch granule-bound protein of 100 kDa (Denyer et al., 1995). The starch hydrolyzing enzymes, α-amylase and β-amylase are of significance in starch quality and can modify amylose/amylopectin ratio. The production of α-

amylase-1 is controlled by genes on the long arms of group 6 chromosomes, and that of α-amylase-2 is by genes on the group 7 long arms (Gale et al., 1995). While the homoeologous β*-amy-1* loci locate on group 4 chromosomes, β*-amy-A1* is in the translocated segment on 5A from 4A. Furthermore, two sucrose synthase loci, *Sus1* and *Ss1*, have been mapped on the short arm of the homoeologous group 7 (Devos et al., 1994). Since the *Wx* locus is flanked with *Sus1* and *Ss1*, the long arm of 4A can localize the sucrose synthase loci on the translocated segment from 7BS. This evidence indicates that *QAmc.ocs-4A.1* on the short arm is independent of known genes affecting starch quality or sucrose synthesis. Whether *QAmc.ocs-4A.1* encodes enzyme(s) in the starch synthesis pathway or acts as a regulator gene should be investigated.

In our population, *QEet.ocs-4A.1*, closely linked to *Wx-B1*, was identified via its effect on flowering time. Hoogendoorn (1985) reported an earliness *per se* factor on chromosome 4A but any linkage with *Wx-B1* is unclear. In barley, a significant linkage of an earliness *per se* gene (*eps7S*) with the *Wx* locus has been implied (Backes et al., 1995; Laurie et al., 1995). Comparative mapping of wheat, barley and rye using molecular markers shows that even if translocations are taken into account, the order of genes including QTLs is conserved on homoeologous chromosome segments (e.g. Laurie, 1997). This predicts a possible relationship between *QEet.ocs-4A.1* and barley *eps7S*, but few common markers exist between the QTL studies and thus it is at present difficult to assess the synteny of these genes. The region adjacent to the *Wx-B1* locus also associated with plant height. The position of this putative *QHt.ocs-4A.1* is contained within a cluster of *Wx-B1* and *QEet.ocs-4A.1*. While Bezant et al. (1997) located a barley plant height QTL (*QHt.psb-7H*) on the distal part of *Wx* locus, no QTLs for plant height have been reported in the vicinity of the *Wx* genes in other Triticeae species.

Although phenotypic differences conditioned by *QEet.ocs-4A.1* and *QHt.ocs-4A.1* are small, compared to the effects of the major *Vrn* or *Ppd* genes on flowering time and those of *Rht* genes on plant height respectively, allelic difference at *QEet.ocs-4A.1* can lead to about 3.0 days variation for flowering time and alleles at *QHt.ocs-4A.1* a 10 cm change in plant height (Table 2). So these QTLs located in the adjacent regions of *Wx-B1* could have breeding potential. If this is the case, the expression of the Wx-B1 protein might be used to select for these traits. Conversely, selection for the *Wx-B1b* allele because of the effects on starch properties may also lead to inadvertent selection for heading date and plant height. In this regard, Miura and Tanii (1994) demonstrated that lack of the Wx proteins can be identified readily using a half endosperm in an SDS-PAGE system. This may permit screening of grains with the null *Wx-B1b* in large populations even in early segregating generations like F_2 and B_1F_1. The selected half grain with the embryo will allow the assessment of the starch properties and morphological traits including flowering time and plant height.

For plant yield and its components, there was no evidence for significant main effects associated with the *Wx-B1* or adjacent regions. One QTL affecting plant yield was detected on the

short arm and a QTL for spikelet number (grain weight/ear) was mapped in a similar position to the plant yield QTL, so it was not enough to explain both the transgressive segregation in the recombinant substitution lines, and the lack of significant differences between the parents. Additional QTLs which would have been able to indicate a correlation with plant yield such as the tiller number QTL might be segregating on chromosome 4A, but were not detected due to lack of appropriate markers. It is also possible that like the QTL for 50 grain weight, the effects of genes could be too weak to be detected clearly within our population. Since all of transgressive segregants detected were the lower-yielding lines compared to the parents, the residual variation on chromosomes other than 4A should be also considered. Hence a possibility of linkage of *Wx-B1* and yield QTLs would not be cancelled out, but even if they occur, such QTL effects are expected to be smaller than the effects of the plant yield QTL, *QYld.ocs-4A.1*, on the short arm. At least in barley, the multiple map compilation by Hayes et al. (1997) shows that no close linkage of yield QTLs with the *Wx* locus on 7HS have been found in five different doubled haploid populations. From this information and our results demonstrated here, we propose that wheat breeders can manipulate the *Wx-B1* locus with giving less attention to yield QTLs.

References

Araki E, Miura H, Sawada S (2000) Differential effects of the null alleles at the three *Wx* loci on the starch-pasting properties of wheat. Theor Appl Genet 100: 1113-1120

Bäckes G, Graner A, Foroughi-Wehr B, Fischbeck G, Wenzel G, Jahoor A (1995) Location of quantitative trait loci (QTL) for agronomic important characters by the use of a RFLP map in barley (*Hordeum vulgare* L.). Theor Appl Genet 90: 294-302

Bezant J, Laurie D, Pratchett N, Chojecki J, Kearsey M (1997) Mapping QTL controlling yield and yield components in a spring barley (*Hordeum vulgare* L.) cross using marker regression. Mol Breeding 3 : 29-38

Chao S, Sharp PJ, Worland AJ, Warhman EJ, Koebner RMD, Gale MD (1989) RFLP-based genetic map of wheat homoeologous group 7 chromosomes. Theor Appl Genet 78: 495-504

Denyer K, Hylton CM, Jenner CF, Smith AM (1995) Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. Planta 196: 256-265

Devos KM, Chao S, Li QY, Simonetti MC, Gale MD (1995) Relationship between chromosome 9 of maize and wheat homoeologous group 7 chromosomes. Genetics 138:1287-1292

Echt C, Schwartz D (1981) Evidence for the inclusion of controlling elements within the structural gene at the *waxy* locus in maize. Genetics 99: 275-284

Gale MD, Atkinson MD, Chinoy CN, Harcourt RL, Jia J, Li QY, Devos KM (1995) Genetic maps of hexaploid wheat. In: Li ZS, Xin ZY (eds) Proc 8th Int Wheat Genet Symp. China Agricultural Science Press, Beijing. pp 29-40

Hayes PM, Cerono J, Witsenboer H, Kuiper M, Zabeau M, Sato K, Kleinhofs A, Kudrna D, Kilian A, Saghai-Maroof M, Hoffman D, the North American Barley Genome Mapping Project (1997) Characterizing and exploiting genetic diversity and quantitative traits in barley (*Hordeum vulgare*) using AFLP markers. J Ag Genomics : http://www.ncgr.org/ag/jag/papers97/paper297/jqtl1997-02.html

Hoogendoorn J (1985) A reciprocal F_1 monosomic analysis of the genetic control of time of ear emergence, number of leaves and number of spikelets in wheat (*Triticum aestivum* L.). Euphytica 34: 545-558

Kato K, Miura H, Akiyama M, Kuroshima M, Sawada S (1998) RFLP mapping of the three major genes, *Vrn1*, *Q* and *B1*, on the long arm of chromosome 5A of wheat. Euphytica 101: 91-95

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12: 172-175

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: An interactive computer package for constructing genetic linkage maps of experimental and natural populations. Genomics 1: 174-181

Laurie DA (1997) Comparative genetics of flowering time. Plant Mol Biol 35:167-177

Laurie DA, Pratchett N, Bezant JH, Snape JW (1995) RFLP mapping of five major genes and eight quantitative loci controlling flowering time in a winter x spring barley (*Hordeum vulgare* L.) cross. Genome 38: 575-585

Law CN (1966) The locations of genetic factors affecting a quantitative character in wheat. Genetics 53: 487-498

Miura H, Tanii S (1994) Endosperm starch properties in several wheat cultivars preferred for Japanese noodles. Euphytica 72: 171-175

Miura H, Sugawara A (1996) Dosage effects of the three *Wx* genes on amylose synthesis in wheat endosperm. Theor Appl Genet 93: 1066-1070

Miura H, Tanii S, Nakamura T, Watanabe N (1994) Genetic control of amylose content in wheat endosperm starch and differential effects of three *Wx* genes. Theor Appl Genet 89: 276-280

Miura H, Tarui S, Araki E, Nakagawa Y (1998) Production of Wx-protein deficient lines in wheat cv. Chinese Spring. In : Slinkard AE (ed), Proc 9th Int Wheat Genet Symp 4, Univ. Exten. Press, Univ. of Saskatchewan, Canada, pp 208-210

Moss HJ (1979) The pasting properties of some wheat starches free of sprout damage. In: Gale MD, Stoy V (eds), Proc 2nd Int Symp Preharvest Sprouting in Cereals, England. pp 297-302

Murray MG, Thompson WF (1980) The isolation of high molecular weight plant DNA. Nucleic Acids Res 8: 4321-4325

Nakamura T, Yamamori M, Hirano H, Hidaka S (1993a) Identification of three Wx proteins in wheat (*Triticum aestivum* L.). Biochem Genet 31: 75-86

Nakamura T, Yamamori M, Hirano H, Hidaka S (1993b) Decrease of waxy (Wx) protein in two common wheat cultivars with low amylose content. Plant Breeding 111: 99-105

Nelson JC, Sorrells ME, Deynze AEW, Lu YH, Atkinson M, Bernard M, Leroy P, Faris JD, Anderson JA (1995) Molecular mapping of wheat: Major genes and rearrangements in homoeologous groups 4, 5 and 7. Genetics 141: 721-731

Oda M, Yasuda Y, Okazaki S, Yamaguchi Y, Yokoyama Y (1980) A method of flour quality assessment for Japanese noodles. Cereal Chem 57: 253-254

Rahman S, Kosar-Hashemi B, Samuel MS, Hill A, Abbot DC, Skerritt JH, Preiss J, Appels R, Morell MK (1995) The major proteins of wheat endosperm starch granules. Aust J Plant Physiol 22: 793-803

Sears ER (1954) The aneuploids of common wheat. Res Bull Univ Mo. USA 572: 1-59

Tinker NA, Mather DE (1995) Methods for QTL analysis with progeny replicated in multiple environments. JQTL 1: http://probe.nalusda.gov:8000/otherdocs/jqtl/jqtl1995-01/jqtl15.html

Toyokawa H, Rubenthaler GL, Powers JR, Schanus EG (1989) Japanese noodle qualities. II. Starch components. Cereal Chem 66: 387-391

Tsai CY (1974) The function of the *waxy* locus in starch synthesis in maize endosperm. Biochem Genet 11: 83-96

Yamamori M, Nakamura T, Kuroda A (1992) Variations in the content of starch-granule bound protein among several Japanese cultivars of common wheat (*Triticum aestivum* L.). Euphytica 64: 215-219

Yamamori M, Nakamura T, Endo TR, Nagamine T (1994) Waxy protein deficiency and chromosomal location of coding genes in common wheat. Theor Appl Genet 89: 179-184

Zhao XC, Batey IL, Sharp PJ, Crosbie G, Barclay I, Wilson R, Morell MK, Appels R (1998) A single genetic locus associated with starch granule properties and noodle quality in wheat. J Cereal Sci 27: 7-13

Table 1 Mean performance of parents, mean and range in the recombinant lines for the agronomic traits

	Ear emergence time (days)[a]	Plant height (cm)	Plant yield (g)	Spikelet no. /ear	Grain wt. /ear (g)	Tiller no. /plant	50 grain weight (g)
1996 season							
Parents							
CS	13.4 ***	109.2 **	10.4	13.1	1.16	17.8 *	1.66
CS*11/Kanto107 4A	10.8	101.1	9.2	14.6	1.19	13.7	1.59
Recombinant lines							
mean	12.6	101.9	8.4	12.3	0.99	14.6	1.62
range	10.8-14.8	91.5-110.7	4.7-12.8	9.8-15.7	0.64-1.34	11.7-19.8	1.45-1.77
1997 season							
Parents							
CS	12.6 ***	114.4 **	15.2	15.1 *	1.42	14.1	1.79
CS*11/Kanto107 4A	9.6	105.4	13.9	16.3	1.49	14.8	1.71
Recombinant lines							
mean	12.8	106.5	12.5	15.1	1.32	12.9	1.71
range	10.0-15.2	95.9-120.1	7.4-17.6	12.9-17.5	0.94-1.65	9.7-18.1	1.54-1.89

[a] ; Days from the 1st July.

Significant levels ; *=0.05-0.01, **=0.01-0.001, ***<0.001.

Table 2 Location of QTLs affecting the agronomic traits

Trait	Locus	Trial	Marker interval	Test statistic	r^2	Additive
Ear emergence time	*QEet.ocs-4A.1*	1996	*Wx-B1*	46.0	0.37	1.2 c
		1997	*Wx-B1*	44.0	0.36	1.6 c
Plant height	*QHt.ocs-4A.1*	1996	*Xpsr119/Wx-B1*	33.5	0.29	4.8 c
		1997	*Xpsr119/Wx-B1*	29.6	0.27	5.7 c
	QHt.ocs-4A.2	1996	*Xbcd1738/Hd*	30.8	0.20	4.5 c
		1997	*Xbcd1738/Hd*	22.6	0.26	4.6 c
Plant yield	*QYld.ocs-4A.1*	1996	*Xbcd1738*	31.1	0.27	1.4 k
		1997	*Xbcd1738*	18.5	0.17	1.7 k
Spikelet no./ear	*QSpn.ocs-4A.1*	1996	*Xbcd1738*	71.1	0.52	1.8 k
		1997	*Xbcd1738*	60.9	0.46	1.4 k
Grain wt./ear	*QGwe.ocs-4A.1*	1996	*Xbcd1738*	31.4	0.27	0.14 k
		1997	*Xbcd1738/Xcdo1387*	12.9	0.12	0.09 k
Tiller no./plant	*QTn.ocs-4A.1*	1996	*Xpsr163*	9.9	0.10	1.0 k
		1997	*Hd/Xpsr163*	17.1	0.16	1.2 k
50 grain weight	*QFgw.ocs-4A.1*	1996	*Xbcd265/Xbcd1738*	18.3	0.17	0.06 k

Additive ; indicates an additive SIM main effect of the parent contributing to a higher value allele, where c=CS and k=CS*11/Kanto107 4A.

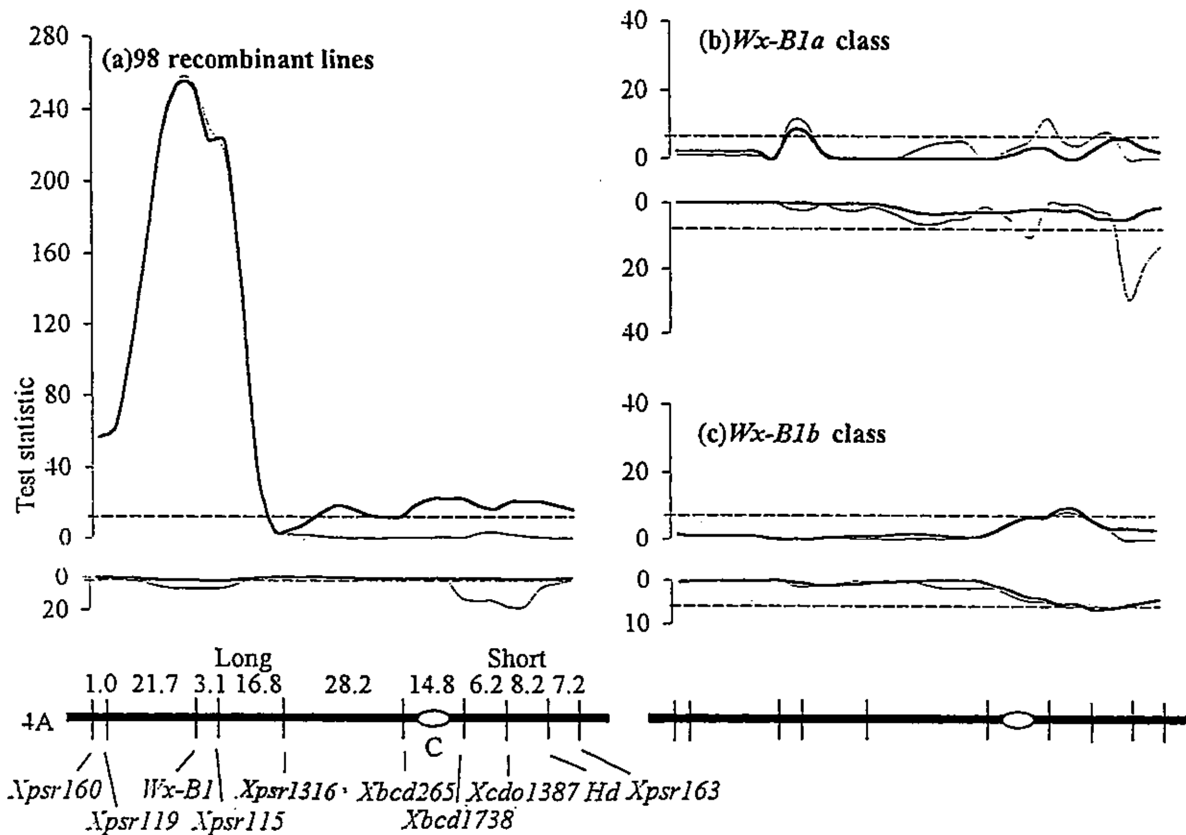


Fig. 1 Effects and posotions of QTLs for amylose content detected by MQTL analysis using all of 98 recombinant lines (a), 47 lines of the *Wx-B1a* class (b) and 51 lines of the *Wx-B1b* class (c). The QTL main effect (upper) and the QTL x season interaction (lower) calculated by both of Simple Interval Mapping (*bold line*) and simplified Composite Interval Mapping (*normal line*) are presented. The *dotted line* indicates the 5% significant threshold level for SIM. The horizontal black bar in the bottom represents the chromosome 4A with both markers and map distances in cM. C ; Centromere.

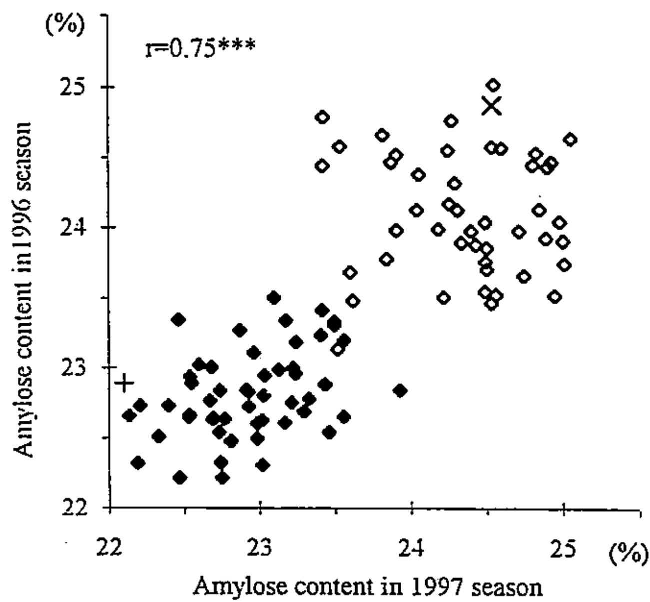
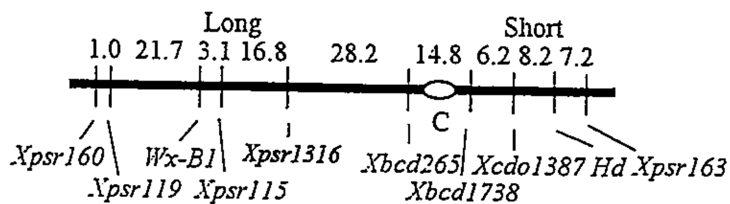


Fig. 2 Variation for amylose content in 98 recombinant lines for chromosome 4A in 1996 and 1997 seasons. × ; Chinese Spring, + ; CS(Kanto107 4A), ◇ ; 47 lines with the *Wx-B1a* allele, ◆ ; 51 lines with the *Wx-B1b* allele. Significant level ; ***P<0.001.



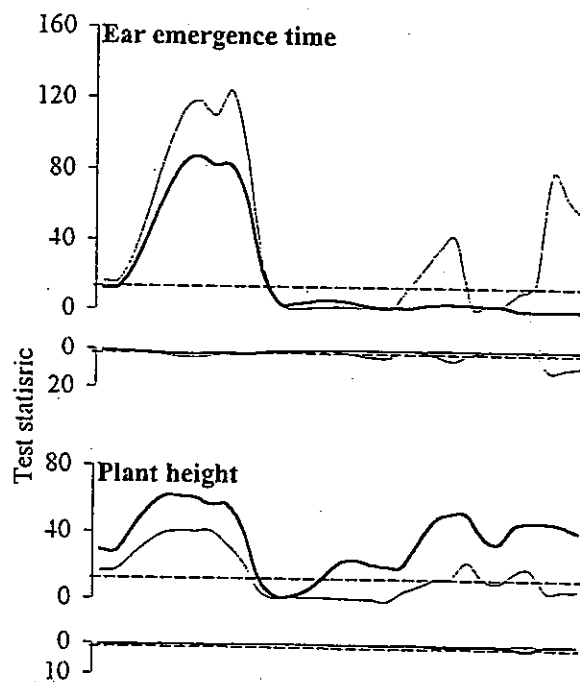


Fig. 3 Effects and positions of QTLs for ear emergence time (top) and plant height (bottom) detected by MQTL analysis on chromosome 4A. The definition of *lines* is the same as in Fig. 1.

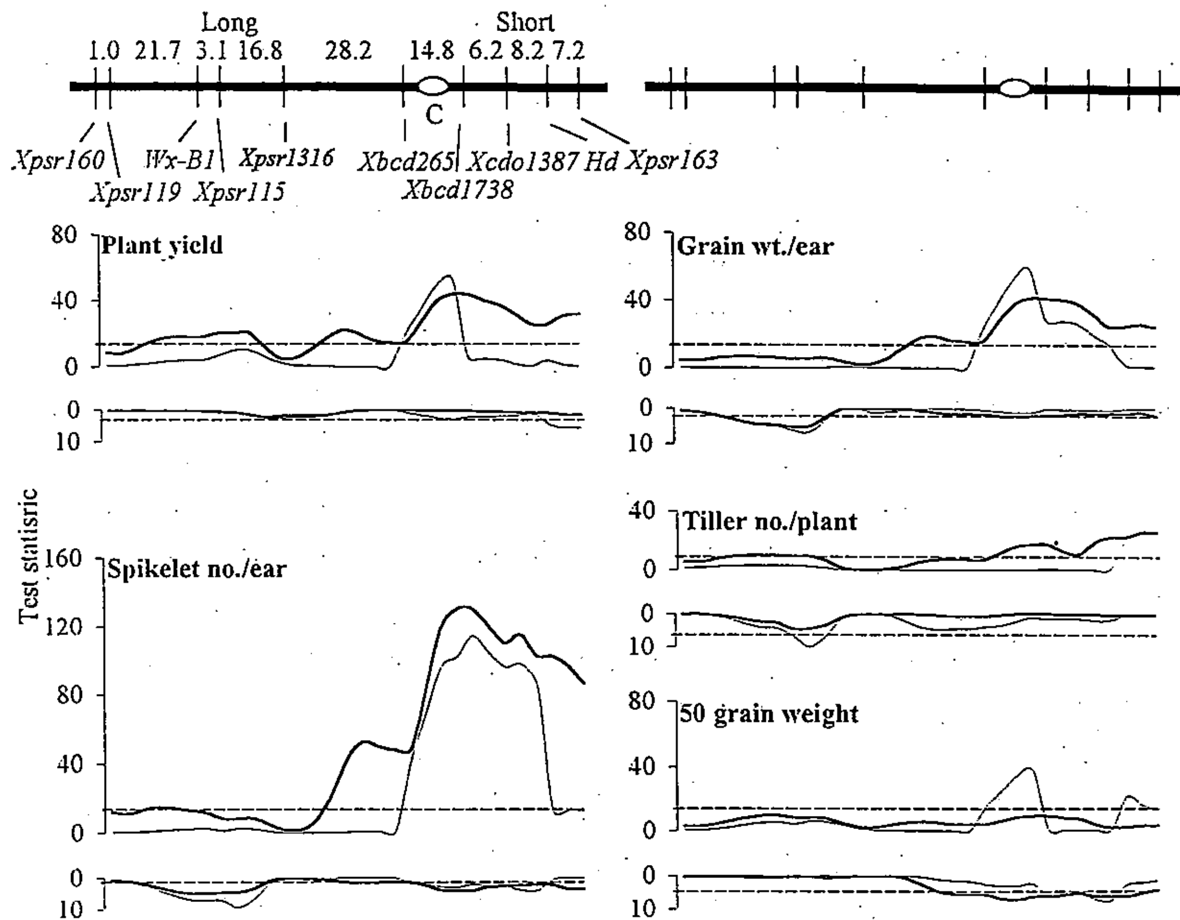


Fig. 4 Effects and positions of QTLs for plant yield and its four component traits detected by MQTL analysis on chromosome 4A. The definition *lines* is the same as in Fig. 1.

Chapter 5. Differential effects of null mutation at the three *Wx* loci on starch pasting properties in wheat

Abstract

The amylose/amylopectin ratio and pasting properties of wheat starch are important in producing marketable flour products, especially Japanese noodle. To determine if the effects of the null mutations at the three *Wx* loci confer categorical differences in starch pasting viscosity, we analyzed variation associated with the null mutations in the three separate sets of single-chromosome recombinant lines for chromosomes 7A, 4A and 7D produced from the crosses between Chinese Spring and three single-chromosome substitution lines carrying the null *Wx* alleles. Differential effects of null alleles at the three *Wx* loci on starch pasting properties were revealed. With respect to chromosome 4A, the pleiotropic effect of the *Wx-B1b* allele on a higher peak and breakdown viscosity was unambiguous. In addition, a QTL of minor effect was identified in near centromere on the short arm. The presence or absence of the Wx-A1 protein can give some variation for peak and breakdown viscosity, but the effects of the null *Wx-A1b* are much smaller compared to those of the *Wx-B1* locus. Associated effects of the *Wx-D1* locus were detected for the breakdown viscosity as the null *Wx-D1b* allele produced a higher viscosity than the wild-type *Wx-D1a*. While negative correlations between amylose content and breakdown viscosity were common in the three populations, the null mutations at the *Wx* loci produced some variations independent of amylose content. The genetic variations detected for breakdown viscosity were more evident than those for peak viscosity in all of three chromosome populations.

Key words; *Triticum aestivum*, amylose content, single-chromosome recombinant, starch pasting property, *Wx* gene

Introduction

The wheat (*Triticum aestivum* L.) endosperm starch normally contains about 20-30% amylose, the rest being amylopectin. Amylose consists of linear molecules of α (1-4)-linked α-D-glucopyranosyl units, whereas amylopectin is made up of highly branched molecules of α-D-glucopyranosyl units linked primarily by (1-4) bonds with branches resulting from α (1-6) linkages. Two distinct types of starch synthases, isoforms of granule-bound starch synthase (GBSS) and soluble starch synthases, involve in the conversion of ADPglucose to the starch polymers. The major GBSS I with a molecular weight of about 60 kDa, so called the Waxy (Wx) protein, is responsible for amylose production. The soluble starch synthases act together with branching enzymes to synthesize amylopectin. Three isoforms of the wheat Wx proteins, Wx-A1, Wx-B1 and Wx-D1, have been

identified by 2D-PAGE (Nakamura et al. 1993a). They are encoded by homoeologous three *Wx* loci, *Wx-A1*, *Wx-B1* and *Wx-D1*, located on 7AS, 4AL and 7DS, respectively (Chao et al. 1989; Nakamura et al. 1993a). Yamamori et al. (1994) have found the null alleles in various cultivars for each of the three controlling loci. A correlation between the presence of null *Wx* alleles and lower amylose content across cultivars has been noticed (Yamamori et al. 1992; Miura and Tanii 1994). The three *Wx* genes have different effects on modifying amylose content, and the null *Wx-B1b* allele provides the largest reduction in the amylose through the lack of the Wx-B1 protein in comparison with the other null *Wx-A1b* and *Wx-D1b* (Miura et al. 1994; Miura and Sugawara 1996). The wild-type *Wx-B1a* predominates for the amylose synthesis capacity, followed by *Wx-D1a* and *Wx-A1a* (Miura et al. 1999).

The amylose/amylopectin ratio of wheat starch is extremely important in producing marketable flour products. The flour from cultivars with lower amylose contents due to the null *Wx-B1b* allele have a higher Japanese noodle quality (Yamamori et al. 1992; Nakamura et al. 1993b; Miura and Tanii 1994; Zhao et al. 1998). It is also agreed that the quality of Japanese noodle is related to starch pasting properties, which involved in starch gelatinization, pasting, and gelation processes (Nagao et al. 1977; Moss 1980; Oda et al. 1980; Toyokawa et al. 1989). Wheat flour with high swelling volume and swelling power (Crosbie 1991; McCormick et al. 1991), high peak viscosity (Moss 1980; Lee et al. 1987; Crosbie et al. 1990), low gelatinization temperature (Oh et al. 1985; Endo et al. 1988) and high rate of breakdown during viscoamylography (Oda et al. 1980; Konik et al. 1992) is desirable for high quality of white salted noodle. Therefore, in addition to a low amylose content, starch properties for processing and eating quality should include higher starch pasting characteristics.

Starch pasting properties of wheat flour have genetic variation (McCormic et al. 1995; Miura and Tanii 1995; Zeng et al. 1997). Generally, a lower amylose content corresponds to higher peak paste viscosity. Zeng et al. (1997) have refered that reduced quantity of starch amylose due to decreased GBSS profoundly affect starch gelatinization, pasting, and gelation properties. On the other hand, there is nearly a continuous spectrum of variation for the viscosity between wild-type and mutant genotypes (Udall et al. 1999), and there are cultivars showing high scores in both amylose content and peak viscosity (Miura and Tanii 1994). These facts argue against a model that a lower amylose content produced by the null mutation is always responsible for a higher peak viscosity. Zhao et al. (1998) provided clear evidence for the genetic association of the null *Wx-B1b* allele with a high peak viscosity, and suggested that the molecular basis for this association is not simply due to decrease in amylose content. Further, although associated effects of chromosomes 7A and 7D on peak viscosity were found (Miura and Tanii 1995; Watanabe and Miura 1997), the effects of the null alleles at the *Wx-A1* and *Wx-D1* loci have not yet been studied in detail.

To determine if the effects of the null *Wx* mutations at the three *Wx* loci confer categorical

differences in starch pasting viscosity, we developed three separate sets of single-chromosome recombinant lines for chromosomes 7A, 4A, and 7D, and analyzed variation associated with the null mutations for starch properties. In the present paper, we report the different effects of the null alleles at the three *Wx* loci on starch pasting properties, especially peak viscosity and breakdown viscosity. Further we mapped quantitative genetic variation on chromosome 4A.

Materials and methods

Plant materials

Previously we developed single-chromosome substitution lines for chromosomes 7A, 4A and 7D with the Chinese Spring (CS) genetic background (Miura and Sugawara 1996). CS carries the *Wx-A1a*, *Wx-B1a* and *Wx-D1a* alleles and thus can produce all of the three Wx proteins. The chromosome 7A substitution line, CS*11/Kanto107 7A, lacks Wx-A1 protein by the *Wx-A1b* allele. Similarly, CS*11/Kanto107 4A carrying *Wx-B1b* does not produce Wx-B1 protein and CS*8/Bai Huo 7D with the null *Wx-D1b* allele has no GBSS I activity to produce Wx-D1 protein.

Using the procedures described by Law (1966), the three sets of homozygous single-chromosome recombinant lines (SCRs) for chromosomes, 7A, 4A and 7D, were developed separately from the F_1s between CS and each of the single-chromosome substitution lines. The development of SCRs for chromosome 4A was already described by Araki et al. (1999). In total 101 different SCRs for chromosome 7A, 95 SCRs for 4A and 105 SCRs for 7D were included in the present experiment. Since chromosome 5D of CS carries the dominant *Vrn-D1* allele promoting spring growth habit (Pugsley 1972), all SCRs and the parental substitution lines are spring wheats.

Identification of the *Wx* allele types

To classify the SCRs for the *Wx* allele types, electrophoretic analysis of starch granule-bound protein was performed. Starch granule preparation and SDS-PAGE were conducted as described by Nakamura et al. (1993a), with the modification that a 15% SDS polyacrylamide gel with an acrylamide/BIS concentration of 30:0.135 was used for electrophoresis.

Starch preparation

Spring sowing trials were conducted in the experimental field of Obihiro University of Agriculture and Veterinary Medicine. The SCR-4A population and the parental genotypes were grown in 1997, and the SCR-7A and SCR-7D populations were examined in 1998. In the second year, CS and the three single-chromosme substitution lines were included. Each genotype was represented by a single plot of 15 plants, spaced 10cm between plants within a row and 30cm between rows. After anthesis, the experimental plots were covered to prevent pre-harvest sprouting. Grain samples harvested were milled on a Brabender Quadrant Junior Test Mill to a final extraction rate of a 60%. Starch granules

were separated using conventional methods.

Amylose content and starch pasting properties

The amylose content per 100mg of starch granules was colorimetrically determined using the Auto Analyzer System II (Bran+Lubbe Co.) as described by Miura et al. (1994). The assessment was carried out at least twice.

Starch peak viscosity and breakdown viscosity were measured on the Rapid Visco Analyzer (RVA, Newport Scientific Pty. Ltd.). 3g of starch was mixed with 25 ml distilled water. The suspension was heated from 55℃ to 95℃ at the rate of 5℃/min and held at 95℃ for 5min, then cooled to 55℃ at the rate of 5℃/min. The primary starch pasting viscosity parameters derived from RVA curve included peak viscosity and minimum viscosity (the lowest viscosity after the hold at 95 ℃). Using these parameters breakdown viscosity was calculated as peak minus minimum viscosity. The analysis was made at least twice. Peak viscosity and breakdown viscosity were measured in Rapid Visco Units (RVU).

Data analysis

Analyses of variance (ANOVA) were performed for starch property data to detect differences between parental genotypes and to partition the variation between SCRs in each chromosome population. A one-way ANOVA was also employed for each *Wx* locus to detect significant differences between the allele class means by comparing them with the variation between lines within classes.

Detection of QTLs on chromosome 4A

Previously, we have constructed the linkage map of chromosome 4A containing *Wx-B1* and other nine markers from an initial population of 98 SCRs (Araki et al. 1999). This map was utilized for the determination and localization of the QTLs for starch pasting properties. QTL analysis was performed using the software package MQTL (Tinker and Mather 1995). The data sets of peak viscosity and breakdown viscosity were analysed by the simple interval mapping (SIM) and simplified composite interval mapping (sCIM) procedures. The linkage group was scanned at a 5-cM interval test statistic. Seven even-spaced background markers were specified for sCIM, and type-I 5% significance thresholds were estimated with 1000 permutations.

Results

Classification of the *Wx* allele types

Of the 95 SCRs for chromosome 4A, 44 lines were found to produce the Wx-B1 protein by the SDS-PAGE system, thus classified as the CS type with *Wx-B1a*. The remaining 51 lines were

deficient in the Wx-B1 protein and identified as the CS*11/Kanto107 4A type with *Wx-B1b*. Of the 101 SCRs for chromosome 7A, 49 lines were identified as the CS type with *Wx-A1a*, and 52 lines identified as the CS*11/Kanto107 7A type with *Wx-A1b*. Of the 105 recombinant lines for chromosome 7D, 48 lines were classified as the CS type with *Wx-D1a*, and 57 lines identified as the CS*8/Bai Huo 7D type with *Wx-D1b*. All of the SCR populations showed non-distorted segregation from the expected 1:1 ratio. This indicated that there was no genetic or genotypic selection in favor of SCRs carrying the particular allele at the each *Wx* locus.

Amylose content

The mean performance of parental genotypes, together with the mean and range in the three sets of SCRs for amylose content and starch pasting properties is given in Table 1. Starch granules from CS produced about 25.0% of amylose over two years. CS*11/Kanto107 4A showed a significantly lower content by about 2.0% than CS. In the SCR-4A, the amylose content of lines with *Wx-B1a* ranged from 23.3% to 25.0%, while the lines with *Wx-B1b* ranged from 22.2% to 23.5%. The null *Wx-B1b* SCRs as a group exhibited a significantly lower amylose content by more than 1% compared to those that produced Wx-B1 protein. The deficiency of the Wx-B1 protein due to the null *Wx-B1b* allele caused a clear reduction in the amylose content.

CS*11/Kanto107 7A and CS*8/Bai Huo 7D showed a significantly lower content by about 1.0% than CS. The ranges of amylose content were almost similar in the SCRs for chromosomes 7A and 7D, as being from 22.7 to 25.5% in the SCR-7A and from 23.0 to 25.9% in the SCR-7D. About 1% amylose difference was detected between the allele class means in each chromosome population. ANOVA revealed highly significant differences between SCRs in each population and the most part of the line variation was explained by the allelic differences at the *Wx-A1* and *Wx-D1*, respectively. Thus, likewise the *Wx-B1* locus, the null alleles at the *Wx-A1* and *Wx-D1* loci were found to have large effects on reduction in the amylose content.

Peak viscosity

The peak viscosity of CS*11/Kanto107 4A was about 15RVU higher than that of CS. The 95 SCR-4A obtained from the CS x CS*11/Kanto107 4A cross showed a large variation ranging from 191 to 302RVU. When the SCRs were separated into the *Wx-B1a* and *Wx-B1b* allele classes, the *Wx-B1b* class had a larger variation while no SCRs in the *Wx-B1a* class produced more than 260RVU in the 1997 trial (Fig. 1a). ANOVA indicated a significant difference between the allele class means at the *Wx-B1* locus, and peak viscosity was negatively correlated with amylose content ($r=-0.46$, $P<0.001$), suggesting an associated effect of the *Wx-B1* locus on peak viscosity.

Among the parental substitution lines in the 1998 experiment, CS*11/Kanto107 7A showed a low peak viscosity of 195RVU which did not differ widely from CS. The 178-248RVU range in the

101 SCRs for chromosome 7A was much smaller than that detected in the SCR-4A and SCR-7D populations (Fig. 1b). The SCRs in the *Wx-A1a* and *Wx-A1b* allele classes showed similar ranges in each other and there was no significant difference between the class means of peak viscosity. A lower amylose content in the *Wx-A1b* allele class was not correlated with a higher peak viscosity (r = -0.14).

CS*8/Bai Huo 7D exhibited a high peak viscosity of 318RVU which differed largely from that of CS. Of the 105 SCRs derived from the CS x CS*8/Bai Huo 7D cross, 57 SCRs in the *Wx-D1b* allele class as a group produced a significantly higher peak viscosity (296RVU) than the *Wx-D1a* allele class of 48 SCRs (267RVU). However, the distributions of the SCRs in these two classes were almost overlapped and thus the reduction in amylose content in the SCRs with *Wx-D1b* did not bring about high peak viscosity (r = 0.01, Fig. 1c).

Breakdown viscosity

Associated effects of the *Wx-B1* locus on breakdown viscosity were more appreciable compared to those on peak paste viscosity. Over two years, starch from CS*11/Kanto107 4A had about 150RVU breakdown viscosity which was consistently higher than those from CS. In the SCR-4A population, a significant difference between the allele class means was revealed. The mean value of around 150RVU in the *Wx-B1b* class was almost equal to the breakdown viscosity of CS*11/Kanto107 4A, as well as that in the *Wx-B1a* class corresponded to about 110RVU breakdown viscosity of CS. The 95 SCRs showed a clear bimodal distribution of breakdown viscosity. The variation in this population related directly to the allele types at the *Wx-B1* locus (Fig. 2a), since most of high breakdown viscosity SCRs have the null allele while the lower SCRs carry the wild-type allele, resulting in a highly negative correlation between breakdown viscosity and amylose content ($r=-0.69$, $P<0.001$).

CS*11/Kanto107 7A and CS*8/Bai Huo 7D showed breakdown viscosity of 99 and 174RVU, respectively. The SCRs-7A population showed a small variation, ranging from 72 to 135RVU. On the other hand, the 96-190RVU range of breakdown viscosity in the SCR-7D population was relatively large and comparable to the range in the SCR-4A population. As shown in Fig. 2b, a negative and significant correlation between breakdown viscosity and amylose content was detected in the SCR-7A ($r = -0.51$, $P<0.001$). The SCRs carrying the *Wx-A1b* tended to have low amylose content and high breakdown viscosity.

Like the SCR-4A population, associated effects of the *Wx-D1* locus on breakdown viscosity were detected. The 105 SCRs for chromosome 7D showed a clear bimodal distribution (Fig. 2c). Most of high breakdown viscosity SCRs have the null *Wx-D1b* allele while the lower SCRs carry *Wx-D1a*, providing a significant difference of about 35RVU between allele classes. In contrast to peak viscosity, a negative correlation between breakdown viscosity and amylose content was found

(r = -0.33, P<0.001), indicating the effects of the null *Wx-D1b* allele on the reduction in amylose content and the increase in breakdown viscosity.

QTL of starch pasting properties on chromosome 4A

For peak viscosity, two QTLs linked in coupling were identified by both SIM and sCIM (Fig. 3). The major QTL effect was detected in the 21.7 cM *Xpsr119/Wx-B1* interval, close to the latter locus. The allelic difference at this locus accounted for 28% of the SCRs variation, and the higher peak viscosity allele came from CS*11/Kanto107 4A with an additive effect of 30RVU (Table 2). The second QTL of minor effect was detected in the *Xbcd1738/Xcdo1387* interval on the short arm, which explained 24% of the line variation. Again the CS*11/Kanto107 4A allele at this QTL contributed to a high peak viscosity with an additive effects of 26RVU.

As expected, two QTLs for breakdown viscosity were identified and mapped to very similar positions, *Xpsr119/Wx-B1* and *Xbcd1738/Xcdo1387* intervals, to the QTLs for peak viscosity (Fig. 3). At these two QTLs, the high breakdown viscosity alleles came from CS*11/Kanto107 4A with additive effects of 34RVU in the *Xpsr119/Wx-B1* QTL and 22RVU in the *Xbcd1738/Xcdo1387* QTL, which explained 56% and 26% of the variation, respectively (Table 2).

The two QTLs for peak viscosity were presumed to be identical with the breakdown viscosity QTLs. In terms of test statistics, percentage of the variation accounted for and additive effects, the QTL effects detected for breakdown viscosity were more evident than those for peak viscosity.

Discussion

With respect to chromosome 4A, the null *Wx-B1b* allele or the allele from CS*11/Kanto107 4A at the *Xpsr119/Wx-B1* QTL contributed to a lower amylose content, and higher peak and breakdown viscosity which are desirable for noodle quality. Such trait correlations may result either from pleiotropic effects of a single locus or from tight linkage of a locus (loci) controlling the traits. If the effects observed in this research are not due to *Wx-B1* but a tightly linked gene located in the 21.7cM *Xpsr119/Wx-B1* interval, recombinations between *Wx-B1* and another locus (loci) would be expected. However, this possibility is cancelled out by the fact that there were no such recombinant lines that have a combination of the *Wx-B1a* allele with a high peak or breakdown viscosity (Figs. 1a, 2a). In addition, as far as it is known, the gene encoding other proteins for starch metabolism such as soluble starch synthases and starch branching enzymes are not located on chromosome 4A (Denyer et al. 1995; Rahman et al. 1995; Yamamori and Endo 1996; Morell et al. 1997; Nagamine et al. 1997). Hence, it is possible to conclude that a higher peak and breakdown viscosity associated with the *Xpsr119/Wx-B1* interval is due to the pleiotropic effects of the *Wx-B1b* allele.

MQTL analysis indicated that as additive effects, the null *Wx-B1b* allele can contribute to increases in 30 and 34RVU for peak and breakdown viscosity, respectively. These effects may be

associated with the difference in amylose content between the allele classes, since the allelic difference at this locus is a primary factor for amylose content accounting for more than 70% of the variation (Araki et al. 1999). However, the SCRs in the *Wx-B1b* allele class with only small differences in amylose content differed appreciably in the starch pasting viscosity, indicating some variation not accounted for by variation in amylose content. These results are in agreement with the research carried out by Zhao et al. (1998). They pointed out that the molecular basis for the effect of the *Wx-B1* null mutation is not simply due to a decrease in amylose content, but the null mutation most likely causes a subtle change in starch structure. However, within the limits of the assays by Zhao et al. (1998) and the present study, the structural basis for the association is difficult to determine. Instead, our result implies the control of a secondary genetic factor which is unidentified previously. The QTL analysis shown in Fig. 3 revealed a possible QTL effect as a candidate for the secondary factor. The same map position, the *Xbcd1738/Xcdo1387* interval, on the short arm of chromosome 4A was shared by *QAmc.ocs-4A.1* for amylose content (Araki et al. 1999), and a QTL for the starch pasting viscosity. This putative QTL of minor effects might involve in variation within the allele classes, but this issue will require further investigation.

For chromosome 7A carrying *Wx-A1*, a negative and significant correlation between breakdown viscosity and amylose content was detected in the SCR-7A population ($r=-0.51$, $P<0.001$). In addition, Miura and Tanii (1995) found that removal of chromosome 7A produced a reduction in peak viscosity. These results might suggest an associated effect of the absence of the Wx-A1 protein on a higher pasting viscosity. Certainly, the SCRs carrying the *Wx-A1b* tended to have low amylose contents and high breakdown viscosity, but the effects of *Wx-A1b* on elevated viscosity seem unremarkable, as the breakdown viscosity variation in the two allele classes of *Wx-A1* was condensed and not substantial to confer categorical differences in the viscosity between classes. Therefore, it is concluded that even if the presence or absence of the Wx-A1 protein can give some variation for the starch pasting viscosity, the effects of the null *Wx-A1b* are much smaller compared to those of the *Wx-B1b* allele.

Associated effects of *Wx-D1* on breakdown viscosity were clearly detected, while the effects on peak viscosity were failed to appear. Like in the SCR-4A population, a bimodal distribution for breakdown viscosity was found in the SCR-7D populatuin. The SCRs with the null *Wx-D1b* allele as a group produced about 35RVU higher viscosity than the SCRs with *Wx-D1a*. These evidences may demonstrate the contribution of the *Wx-D1* null mutation to a higher breakdown viscosity.

Consequently, as far as this experiment is concerned, we conclude the differential effcts of the null mutations at the three *Wx* loci on starch pasting viscosity.

However, when we discuss the effects of chromosomes 7A and 7D on starch properties, it seems important to consider the co-location on group 7 chromosomes of the genes for enzymes related to starch synthesis. For example, starch branching enzyme (SBE) isolated from wheat

endosperm is fractionated into two distinct isoforms, SBE I and SBE II. Of them, SBE I with approximately 85kDa in molecular mass further consists of several isoforms. The genes controlling some of the SBE I isoforms are assigned on the short arm of the homoeologous group 7 chromosomes (Morell et al. 1997; Nagamine et al. 1997). The genes for ADPglucose pyrophosphorylase have been mapped on chromosome 7 (Devos and Gale 1997). In addition, the minor GBSS with a molecular weight of 10,000-11,500 are also encoded by homoeologous loci on the short arms of group 7 chromosomes (Denyer et al. 1995; Yamamori and Endo 1996), and they are suggested to be soluble starch synthases (Takaoka et al. 1997). The respective roles of SBE I, and the 10,000-11,500 molecular weight proteins in wheat starch synthesis have not yet been unambiguously defined (Morell et al. 1997). Therefore, prior to draw a more precise conclusion about the associated effects of the null *Wx-A1* and *Wx-D1* mutations on starch pasting properties, it remains to be seen if these enzymes except GBSS I affect starch pasting properties and if there are polymorphisms at those loci in our SCR populations for chromosomes 7A and 7D. At present, the lack of sufficient number of polymorphic marker loci hampers the analysis of these chromosomes. In further work, however, the three sets of single-chromosome recombinant populations can have some advantages as a tool for defining genetic variation associated with the other loci mentioned above, and for identifying QTL effects under the uniform genetic background of CS by growing them in different environments.

Any information leading our understanding of genetic mechanisms of the starch pasting properties will improve the breeding process. The results derived from the current experiment have general consequences for strategies for the breeding of wheat cultivars with preferable quality for white noodle. At least, the associated effects of *Wx-B1* were common for amylose content and starch pasting properties, showing that it should be possible to modify several aspects of starch properties simultaneously. This may support an idea that the null *Wx-B1b* is worth selecting not only for a lower amylose content (Araki et al. 1999; Miura et al. 1999), but for a higher starch pasting viscosity in breeding programs (Miura and Tanii 1994; Zhao et al. 1998). Furthermore, Udall et al. (1999) have mapped several quantitative trait loci (QTL) for peak viscosity and these QTL effects are independent of variation due to the *Wx* null mutations because the mapping population used is not segregating for the *Wx* loci. Such QTLs including the *Xbcd1738/Xcdo1387* QTL identified here may have certain breeding potential after the introduction of the null mutation at the *Wx* loci.

References

Araki E, Miura H, Sawada S (1999) Identification of genetic loci affecting amylose content and agronomic traits on chromosome 4A of wheat. Theor Appl Genet 98: 977-984

Chao S, Sharp PJ, Worland AJ, Warhman EJ, Koebner RMD, Gale MD (1989) RFLP-based genetic map of wheat homoeologous group 7 chromosomes. Theor Appl Genet 78: 495-504

Crosbie GB, Miskelly D, Dewan T (1990) Wheat quality for the Japanese flour milling and noodle industries. Aust J Agric 31: 83-88

Crosbie GB (1991) The relationship between starch swelling properties, paste viscosity and boiled noodle quality in wheat flours. J Cereal Sci 13: 145-150

Denyer K, Hylton CM, Jenner CF, Smith AM (1995) Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. Planta 196: 256-265

Devos NM, Gale MD (1997) Homoeologous group 7. In: McGuire PE, Qualset CO (eds) Progress in genome mapping of wheat and related species. (Joint Proc 5th 6th Public Workshops Int Triticeae Mapping Initiative). Genetic Resources Conservation Program, University of California, pp 11-23

Endo S, Karibe S, Okada K, Nagao S (1988) Studies on the noodle making quality of Japanese domestic wheat flours. I. Factors affecting gelatinization properties of wheat starch. Nippon Shokuhin Kogyo Gakkaishi 35: 7-14

Konik CM, Miskelly DM, Gras PW (1992) Contribution of strach and non-starch parameters to the eating quality of Japanese white salted noodles. J Sci Food Agric 58: 403-406

Law CN (1966) The locations of genetic factors affecting a quantitative character in wheat. Genetics 53: 487-498

Lee CH, Gore PJ, Lee HD, Yoo BS, Hong SH (1987) Utilisation of Australian wheat for Korean style dried noodle making. J Cereal Sci 6: 283-297

McCormick KM, Panozzo JF, Hong SH (1991) A swelling power test for selecting potential noodle quality wheats. Aust J Agric Res 42: 317-323

McCormick KM, Panozzo JF, Eagles HA (1995) Starch pasting properties and genetic relation-ship of wheat cultivars important to Victorian wheat breeding. Aust J Agric Res 46: 861-871

Miura H, Tanii S (1994) Endosperm starch properties in several wheat cultivars preferred for Japanese noodles. Euphytica 72: 171-175

Miura H, Tanii S (1995) Chromosomal assignment of genes for the peak paste viscosity of wheat flour using aneuploid lines. J Genet Breed 49: 261-264

Miura H, Sugawara A (1996) Dosage effects of the three *Wx* genes on amylose synthesis in wheat endosperm. Theor Appl Genet 93: 1066-1070

Miura H, Tanii S, Nakamura T, Watanabe N (1994) Genetic control of amylose content in wheat endosperm starch and differential effects of three *Wx* genes. Theor Appl Genet 89: 276-280

Miura H, Araki E, Tarui S (1999) Amylose synthesis capacity of the three *Wx* genes of wheat cv. Chinese Spring. Euphytica (in press)

Morell MK, Blennow A, Kosar-Hashemi B, Samuel MS (1997) Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. Plant Physiol 113: 201-208

Moss HJ (1980) The pasting properties of some wheat starches free of sprout damage. Cereal Res Comm 8: 297-302

Nagamine T, Yoshida H, Komae K (1997) Varietal differences and chromosome locations of multiple isoforms of starch branching enzyme in wheat endosperm. Phytochem 46: 23-26

Nagao S, Ishibashi S, Imai S, Kanbe T, Kaneko Y, Otsubo H (1977) Quality characteristics of soft wheats and their utilization in Japan. III. Effects of crop year and protein content of product quality. Cereal Chem. 54: 198-204

Nakamura T, Yamamori M, Hirano H, Hidaka S (1993a) Identification of three Wx proteins in wheat (*Triticum aestivum* L.). Biochem Genet 31: 75-86

Nakamura T, Yamamori M, Hirano H, Hidaka S (1993b) Decrease of waxy (Wx) protein in two common wheat cultivars with low amylose content. Plant Breeding 111: 99-105

Oda M, Yasuda Y, Okazaki S, Yamaguchi Y, Yokoyama Y (1980) A method of flour quality assessment for Japanese noodles. Cereal Chem 57: 253-254

Oh NH, Seib PA, Ward AB, Deyoe CW (1985) Noodles. IV. Influence of flour protein, extraction rate, particle size, and starch damage on the quality characteristics of dry noodles. Cereal Chem 62: 441-446

Pugsley AT (1972) Additional gene inhibiting winter habit in wheat. Euphytica 21: 21-31

Rahman S, Kosar-Hshemi B, Samuel MS, Hill A, Abbot DC, Skerritt JH, Preiss J, Appels R, Morell MK (1995) The major proteins of wheat endosperm starch granules. Aust J Plant Physiol 22: 793-803

Takaoka M, Watanabe S, Sassa H, Yamamori M, Nakamura T, Sasakuma T, Hirano H (1997) Structural characterization of high molecular weight starch granule-bound proteins in wheat (*Triticum aestivum* L.). J Agric Food Chem 45: 2929-2934

Tinker NA, Mather DE (1995) Methods for QTL analysis with progeny replicated in multiple environments. JQTL 1: http://probe.nalusda.gov:8000/otherdocs/jptl/jptl1995-01/jptl15.html

Toyokawa H, Rubenthaler GL, Powers JR, Schanus EG (1989) Japanese noodle qualities. II. Starch components. Cereal Chem 66: 387-391

Udall JA, Souza E, Anderson J, Sorrells ME, Zemetra RS (1999) Quantitative trait loci for flour viscosity in winter wheat. Crop Sci 39: 238-242

Watanabe N, Miura H (1997) P gene effects on starch pasting properties of tetraploid and hexaploid wheat. Cereal Res Commun 25: 963-968

Yamamori M, Endo TR (1996) Variation of starch granule proteins and chromosome mapping of their coding genes in common wheat. Theor Appl Genet 93: 275-281

Yamamori M, Nakamura T, Kuroda A (1992) Variations in the content of starch-granule bound protein among several Japanese cultivars of common wheat (*Triticum aestivum* L.). Euphytica 64: 215-219

Yamamori M, Nakamura T, Endo TR, Nagamine T (1994) Waxy protein deficiency and chromosomal location of coding genes in common wheat. Theor Appl Genet 89: 179-184

Zeng M, Morris CF, Batey IL, Wrigley CW (1997) Sources of variation for starch gelatinization, pasting, and gelation properties in wheat. Cereal Chem 74: 63-71

Zhao XC, Batey IL, Sharp PJ, Crosbie G, Barclay I, Wilson R, Morell MK, Appels R (1998) A single genetic locus associated with starch granule properties and noodle quality in wheat. J Cereal Sci 27: 7-13

Table 1 Mean performance of parental genotypes, together with the mean and range in the *Wx* allele classes of the three RSL populations for amylose content and starch-pasting properties

Item	No. of RSLs	Amylose content Mean (%)	Amylose content Range (%)	Peak viscosity Mean (RVU)	Peak viscosity Range (RVU)	Breakdown viscosity Mean (RVU)	Breakdown viscosity Range (RVU)
Parental genotypes (1997)							
CS		24.9		240		118	
CS*11/Kanto107 4A		22.9		265		152	
RSLs for chromosome 4A	95						
Wx-B1a	44	24.1	23.3-25.0	221	193-254	111	98-129
Wx-B1b	51	22.8	22.2-23.5	247	191-302	144	100-184
		***		***		***	
Parental genotypes (1998)							
CS		25.1		226		111	
CS*11/Kanto107 4A		23.1		242		142	
CS*11/Kanto107 7A		24.0		195		99	
CS*8/Bai Huo 7D		23.9		318		174	
RSLs for chromosome 7A	101						
Wx-A1a	49	24.7	23.6-25.5	225	178-248	106	72-128
Wx-A1b	52	23.9	22.7-25.2	228	191-248	117	96-135
		***		ns		**	
RSLs for chromosome 7D	105						
Wx-D1a	48	25.0	24.1-25.9	267	212-316	131	96-157
Wx-D1b	57	24.0	23.0-25.2	296	236-330	166	129-190
		***		***		***	

, * ; Significant differences between the allele class means at the 1% and 0.1% levels, respectively.
ns ; non-significant.

Table 2 Location of QTLs on chromosome 4A for starch pasting properties

Trait	Marker interval	r^2	Additive [a]
Peak viscosity	*Xpsr119/Wx-B1*	0.28	30
	Xbcd1738/Xcdo1387	0.24	26
Breakdown viscosity	*Xpsr119/Wx-B1*	0.56	34
	Xbcd1738/Xcdo1387	0.26	22

[a] Additive : indicates an additive SIM main effect, all from Kanto107.

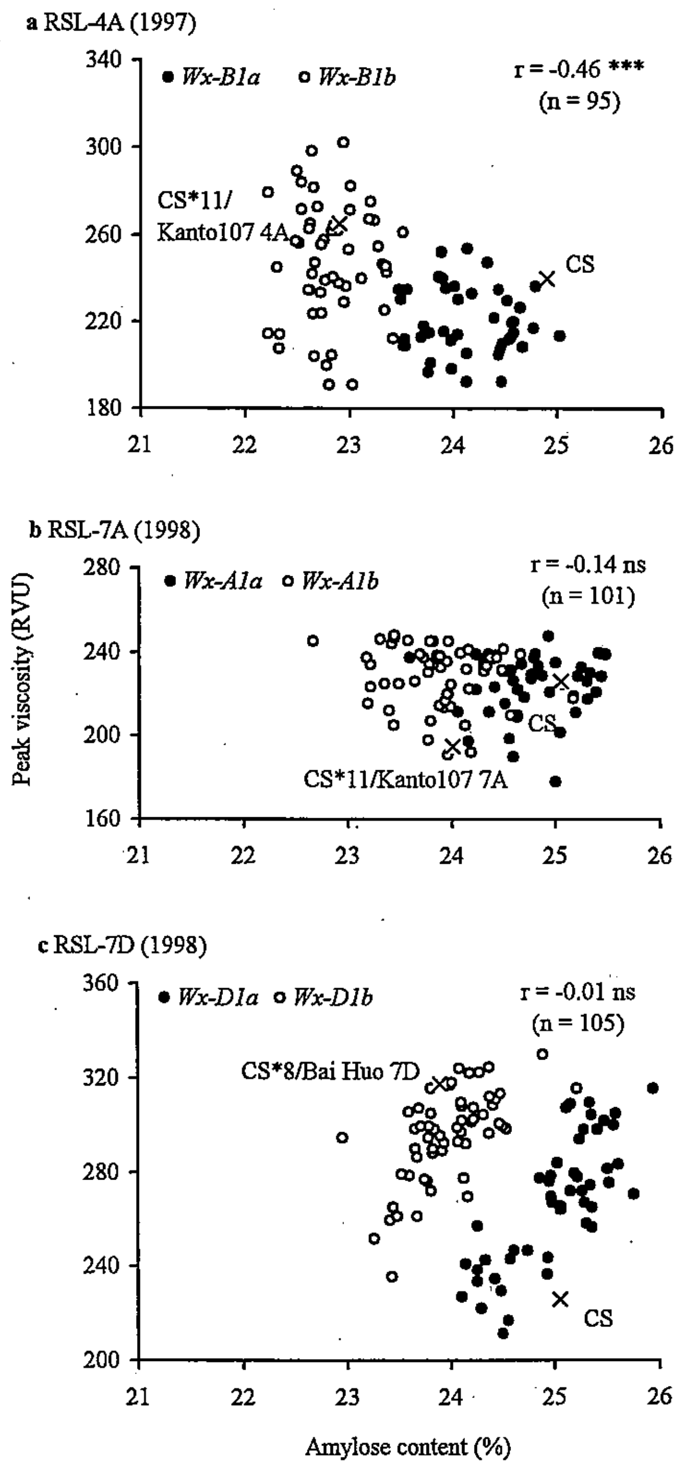


Fig. 1 Relationship between amylose content and peak viscosity of starch in the recombinant substitution lines (RSLs) of chromosomes 4A (a), 7A (b) and 7D (c). ***P<0.001, ns : non-significant.

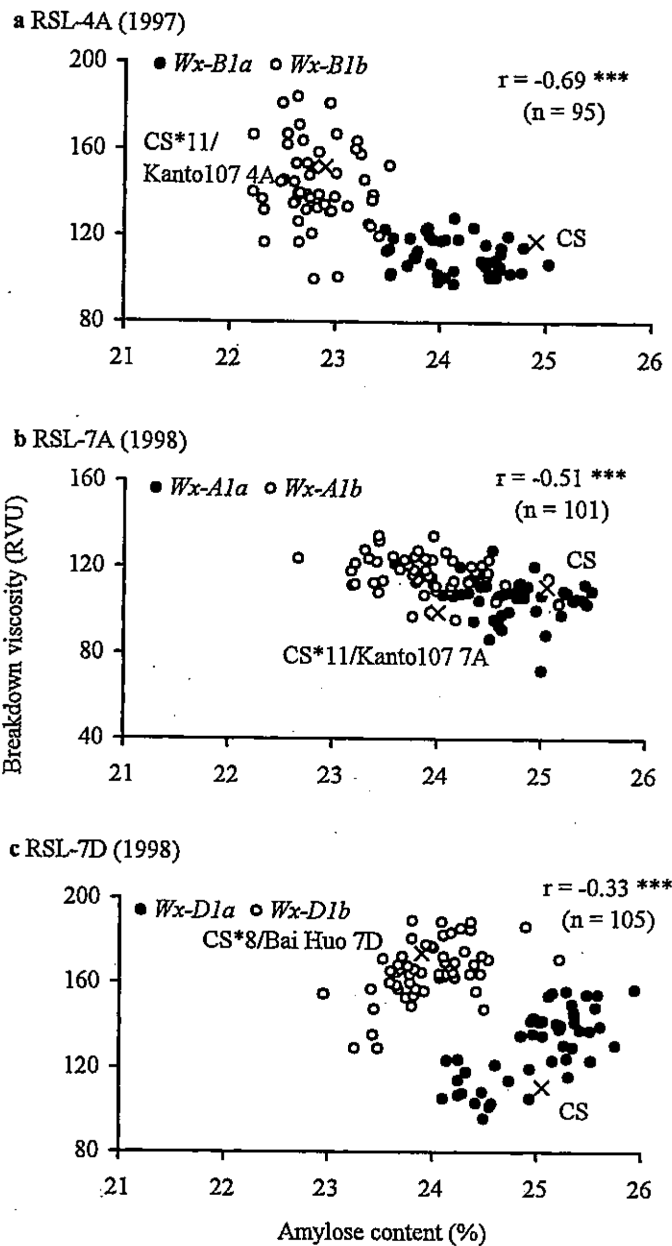


Fig. 2 Relationship between amylose content and breakdown viscosity of starch in the recombinant substitution lines (RSLs) of chromosomes 4A (**a**), 7A (**b**) and 7D (**c**). ***P<0.001.

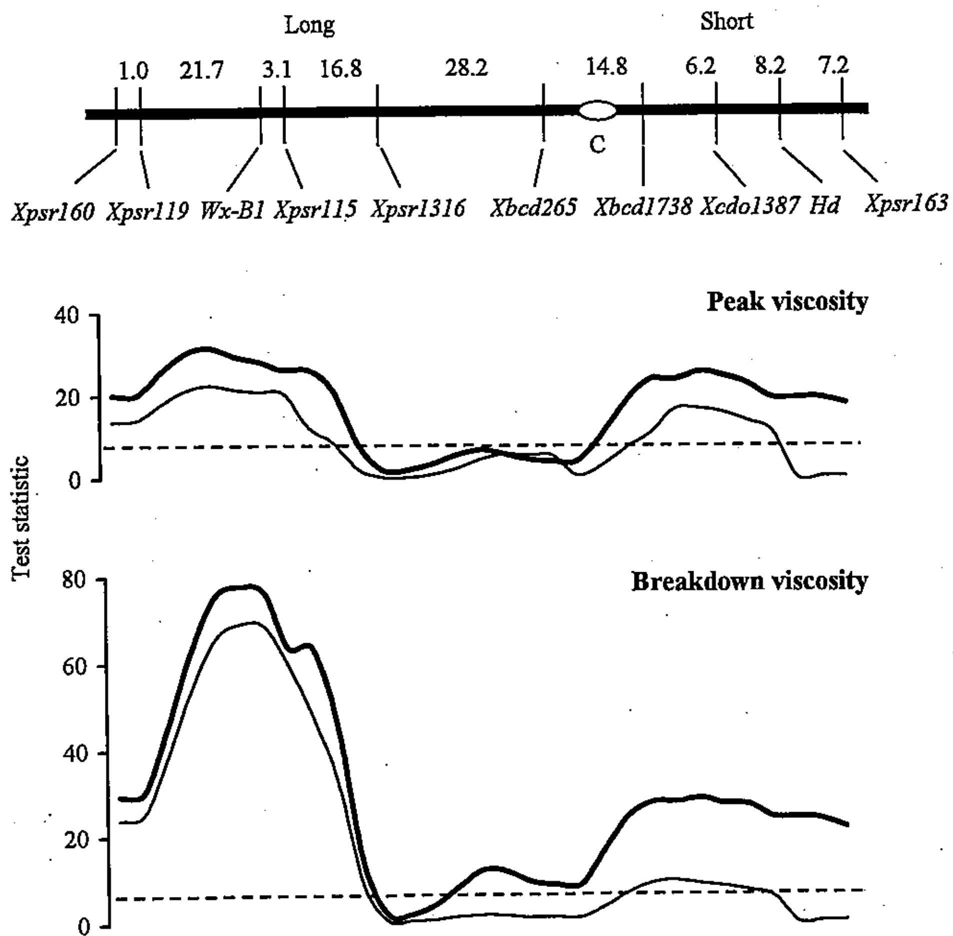


Fig. 3 Effects and positions of QTLs for peak viscosity (top) and breakdown viscosity (bottom) detected by MQTL analysis of chromosome 4A. The QTL main effect calculated by both of Simple Interval Mapping (*bold line*) and simplified Composite Interval Mapping (*normal line*) is presented. The *dotted line* indicates the 5% significance threshold level for SIM. The *horizontal black bar* at the top represents chromosome 4A with the markers, map distances (cM), and centromere (C) indicated.

Summary

The advent of routine genetic manipulation of the enzymes catalyzing starch synthesis in the wheat endosperm has made it possible to change the physical and chemical characteristics of starch and to provide novel variation in raw materials. However, it has only been in the last decade so that wheat scientist and breeder have recognized that genetic variation for starch properties, starch composition in particular, is responsible for the variation in end-use quality. Therefore an improved understanding of the genes coding amylose and amylopectin synthesizing enzymes is needed.

The present study was carried out to define and discuss the contributions of the homoeologous three *Wx* genes of wheat to variation for amylose content, starch structure, starch pasting properties and important agronomic traits. The subjects focused in this study are as follows. Amylose synthesis capacity of the three *Wx* genes was determined in Chapter 2 using eight possible types with different combinations of wild-type and null alleles at the *Wx* loci. Then the amylopectin molecular structures in the eight types were compared in Chapter 3. The contributions of *Wx-B1* on chromosome 4A to agronomic traits, as well as quantitative trait loci (QTL) for amylose content were analyzed in Chapter 4. In Chapter 5, the effects of the null alleles at the *Wx* loci on starch pasting properties were distinguished using three separate sets of single-chromosome recombinant substitution lines for chromosomes 7A, 4A and 7D. The results obtained from this study are summarized as follows.

Amylose synthesis capacity of the three *Wx* genes was compared in Chapter 2. Amylose content varied from 0% of the waxy Chinese Spring (CS) to 25% of the wild-type CS. The reducing effect of the single null alleles was the largest in *Wx-B1b*, and there was no significant difference between *Wx-A1b* and *Wx-D1b*. More than 3% reductions in amylose content from the wild-type were detected in the double null types. The results of the double null lines further demonstrated that for the capacity of amylose synthesis, *Wx-B1a* predominates and produces 21-22 % amylose, followed by *Wx-D1a* (20-21%) and *Wx-A1a* (15-18%). These significant differences were partly correlated with variation in the amounts of the Wx proteins produced by different *Wx* genes. However comparisons of the double null lines with the single null or the wild-type indicated that amylose content was not linearly proportional to the number of the functional *Wx* alleles, suggesting that the wheat *Wx* genes act in an epistatic manner.

For the chain-length distribution analysis of amylopectin structure in the eight possible types of the Wx-protein deficient lines, high-performance anion-exchange chromatography (HPAEC) was used. The results were described in Chapter 3 . HPAEC can separate malto-oligosaccharides according to their degree of polymerization under alkaline conditions. The unit-chain distribution of amylopectin were classified at the inflection points of the elution profiles into three groups. For any of the three groups of degree of polymerization, the HPAEC system detected no clear differences among the eight types of the Wx-protein deficient lines. Thus it was found that the reduced GBSS I

activity due to introgression of the null *Wx* alleles does not affect the degree of branching of amylopectin.

To determine the effects of the *Wx-B1* locus and effects of independent QTLs on agronomic traits, genetical analysis of chromosome 4A was conducted in Chapter 4 using 98 recombinant inbred lines derived from a cross of CS and CS*11/Kanto107 4A having a low amylose content due to the null *Wx-B1b* allele. For amylose content, the most part of the genetic variation was explained by the allelic difference at the *Wx-B1* locus. An additional QTL of a minor effect was mapped in the 6.2 cM *Xbcd1738/Xcdo1387* interval on the short arm, where the allele from Kanto107 increased amylose content. Field trials over two seasons revealed a pleiotropic effect of *Wx-B1* or the effect of a closely linked QTL on ear emergence time. A QTL linked to *Wx-B1* was detected for plant height. For plant yield and its components, there was no evidence for significant main effects associated with *Wx-B1* or adjacent regions. One plant yield QTL was identified by DNA markers on the short arm and this was identical to QTLs controlling spikelet number/ear and grain weight/ear. At these QTLs for agronomic traits, alleles from Kanto107 contributed to an earlier emergence time, height reduction and yield increase.

The amylose content and the pasting properties of wheat starch are important in producing marketable flour products, especially Japanese noodle. To determine if effects of the null mutations at the three *Wx* loci confer categorical differences in starch-pasting viscosity, the genetic variation associated with the null mutations in the three separate sets of recombinant inbred lines for chromosomes 7A, 4A and 7D produced from the crosses between CS and three single-chromosome substitution lines carrying the null *Wx* alleles was analyzed in Chapter 5. Differential effects of null alleles at the three *Wx* loci on starch-pasting properties were revealed. With respect to chromosome 4A, the associated effect of the *Wx-B1b* allele giving a higher peak and breakdown viscosity, was unambiguous. In addition, a QTL of minor effect was identified in near centromere on the short arm. The presence or absence of the Wx-A1 protein can give some variation for peak and breakdown viscosity, but the effects of the null *Wx-A1b* are much smaller than those of the *Wx-B1b* allele. Associated effects of the *Wx-D1* locus were detected for the breakdown viscosity as the null *Wx-D1b* allele produced a higher viscosity than the wild-type *Wx-D1a*. While negative correlation between amylose content and breakdown viscosity was common in the three populations, the null mutations at the *Wx* loci produced some variations independent of amylose content. The genetic variations detected for breakdown viscosity were more evident than those for peak viscosity in all three chromosome populations.

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