MOLECULAR CHARACTERIZATION OF WAXY MUTANTS IN HEXAPLOID WHEAT

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ABSTRACT

Recent research has focused on the molecular characterization of null waxy (Wx) alleles, *Wx-A1b*, *Wx-B1b*, and *Wx-D1b*, that produce no detectable Wx proteins in the endosperm starch of allohexaploid wheat (*Triticum aestivum* L.; 2n=6x=42; AABBDD). The major objectives of this thesis were to (1) isolate and characterize a Wx wheat cDNA and (2) to identify aberrant *Wx* transcripts encoded by the null *Wx-A1b* allele of CDC Wx2, a waxy hexaploid wheat line, which result in an absent Wx-A1 protein (~59 kD). In the first study, a cDNA library prepared from developing wheat kernels (cv. Fielder; *Wx-A1a*, *Wx-B1b*, and *Wx-D1a* alleles) was screened using a homologous PCR-digoxigenin labeled wheat cDNA probe. A 2.2 kb cDNA clone denoted GBSSIMMI (Accession no. Y16340) was sequenced and identified as encoding a Wx-D1 protein. The deduced amino acid sequence showed 94 % similarity with a wheat Wx-A1 peptide, 96 % similarity with a wheat Wx-B1 peptide, and 100 % identity with two wheat Wx-D1 peptides. A 33-nucleotide deletion, encoding 11 amino acids (AMLCRAVPRRA), was detected within the GBSSIMMI cDNA relative to a previously isolated wheat cDNA (accession no. X57233). Complementation analysis using a glycogen synthase deficient *E. coli* strain and an *in vitro* starch synthase assay did not indicate that GBSSIMMI encoded a functional Wx-D1 protein. In the second study, two sister lines CDC Wx2 and CDC Wx6 were obtained by crossing lines Bai-Huo (carries null *Wx-D1b* allele; lacks Wx-D1 protein) and Kanto 107 (carries null *Wx-A1b* and *-B1b* alleles; lacks Wx-A1 and -B1 proteins). Waxy protein profiling, amylose concentration determinations, Northern blot analysis, and reverse transcriptase PCR (RT-PCR) analysis were conducted. Ten RT-PCR derived cDNA clones were selected from each genotype and characterized by DNA sequencing analyses. The waxy phenotype of CDC Wx2, lacking Wx-A1, -B1, and -D1 proteins and possessing a reduced amylose concentration (~ 4 %), was associated with dramatically reduced levels of a 2.4 kb *Wx* transcript when compared to the higher levels in a wildtype control line. DNA sequencing of clones from Kanto 107 and CDC Wx2 characterized two types of aberrant *Wx* transcripts, one containing intron 1 and another containing introns 1 and 4. Intron 1 in both types of aberrant *Wx* transcripts

contained a premature stop codon which resulted in the translation of a truncated Wx protein (~ 4 or 11 kD). Analysis of CDC Wx6, lacking Wx-B1 and -D1 proteins and possessing a reduced amylose concentration (~ 14 %), failed to reveal aberrant *Wx* transcripts, suggesting that the RNA defects in this study were not responsible for the absence of the Wx-B1 or -D1 proteins. Thus, the aberrant *Wx* transcripts were encoded by the null *Wx-A1b* allele. The presence of a premature stop codon in the *Wx* transcripts encoded by the null *Wx-A1b* allele explained the absence of the ~ 59 kD Wx-A1 protein in CDC Wx2 and its parental line Kanto 107.

TO MY LOVING FAMILY

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LIST OF ABBREVIATIONS

AABB	Genomes A and B of tetraploid wheats
AABBDD	Genomes A, B, and, D of hexaploid wheats
AAP	Amorphous amylopectin
-	Absence
A	Absorbance in optical densities
ADP-GPPase	ADP-Glucose pyrophosphorylase
ADPG	ADP-Glucose
Ab	Antibody
Ag	Antigen
BE or SBE	Starch branching enzyme
BEI/BEII	Isoforms I and II of starch branching enzyme
bp	Base pair
BSA	Bovine serum albumin
CTAB	Cetylmethylammonium bromide
cpm	Counts per minute
CAP	Crystalline amylopectin
CDC	Crop Development Centre
cDNA	Complementary deoxyribonucleic acid
cv	Cultivar
DBE	Debranching enzyme
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
2x/4x/6x	Diploid/tetraploid/hexaploid
DPA	Days post anthesis
GBSS	Granule-bound starch synthase
GBSSI/GBSSII	Isoforms I and II of granule-bound starch synthase
G1P	Glucose-1-phosphate
h	Hour
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thio-galactopyranoside
kb	Kilobase
kD	Kilodalton

mRNA	Messanger RNA
28S and 18S	Ribosomal RNA
tRNA	Transfer RNA
min	Minutes
MT	Millions of tonnes
no/nos	Number/numbers
NRCC	National Research Council of Canada
nt	Nucleotides
1D/2D	One dimentional/two dimensional
PBI	Plant Biotechnology Institute
PAM	Percent accepted mutations
PCR	Polymerase chain reaction
+	Presence
pI	Isoelectric point
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Seconds
SS	Starch synthase
SSI/SSII/SSIII	Isoforms I, II, and III of starch synthase
SSS	Soluble starch synthase
Tm	Melting temperature
U1 to U5	Ribonucleoprotein particles
U	Units
V	Volts
Wx	Waxy
Wx-A1	Waxy protein encoded by the *Wx* gene on chromosome 7A
Wx-B1	Waxy protein encoded by the *Wx* gene on chromosome 4A (translocated from 7BS)
Wx-D1	Waxy protein encoded by the *Wx* gene on chromosome 7D
Wx-A1a	Wildtype allele encoding a Wx-A1 protein
Wx-B1a	Wildtype allele encoding a Wx-B1 protein
Wx-D1a	Wildtype allele encoding a Wx-D1 protein
Wx-A1b	Null allele resulting in no detectable Wx-A1 protein
Wx-B1b	Null allele resulting in no detectable Wx-B1 protein
Wx-D1b	Null allele resulting in no detectable Wx-D1 protein

1.0 INTRODUCTION

Wheat (*Triticum* L.), one of the most important food staples of the world's population, is used in baked goods, pasta and noodle products, fish cake (kamaboko), imitation crab legs, rice confectioneries, and dumplings (Oleson, 1994; Maningat and Seib, 1997). Wheat starch is used as a raw material in industrial and non-food end-uses such as carbonless paper, adhesive for corrugated boards, drywall textures and joint cements, cushioning materials, microcellular foams, cosmetics, film, mash fermentation for ethanol production, and alcoholic beverages. Common wheat (*T. aestivum* subsp. *vulgare* [Vill. Host] Mackey; 2n=6x=42, AABBDD) consitutes 95 % of world wheat production whereas durum wheat (*T. durum* Desf.; 2n=4x=28, AABB) accounts for 5 %. In 1992-1996, Canada exported 15 million tonnes (MT) of common wheat and 4 MT of durum wheat (Canadian Wheat Board, personal communication). Canada exports seven classes of high-quality wheats including six common wheats and one durum (Table 1.1). The wheat classes vary in kernel hardness, protein content, and gluten strength which together account for most of the variance in functionality among these classes. These functional differences make each market class suitable for particular end-uses. Generally, high protein hard wheats are favored for bread production, durums are used for pasta, low protein soft wheats are used to produce biscuits, cookies and crackers, and medium protein wheats, hard or soft, are used in Asian noodle applications (Chen, 1993).

In 1992-1996, Asian-Pacific countries imported 33 MT of wheat suggesting that their noodle markets represent significant high value markets for Canadian wheats (Canadian Wheat Board, personal communication). Wheat imports into these countries are projected to rise to 40 MT in the year 2007. In Canada, flours destined for noodle production are made by blending flours originating from all classes of wheat, except Canada Eastern Soft White Winter, Canada Western Extra Strong, and Canada Western Amber Durum (Chen, 1993). A reduced amylose concentration has been shown to dramatically affect the functional properties of wheat starch and thus its suitablility for producing

Table 1.1 Characteristic kernel color, kernel hardness, protein, and gluten strength of five different wheat classes † (Adapted from Williams, 1993).

Wheat class (cultivar)	Kernel color	Kernel hardness	Protein	Gluten strength	End-uses
CWRS[a] (AC Barrie)	Red	Hard	High (13.6 %)	Strong	Long fermentable pan breads
CWES[b] (Glenlea)	Red	Hard	Medium high (12.5 %)	Very Strong	Frozen dough, hearth breads, pan breads
CPS[c] (AC Crystal) (AC Karma)	Red White	Hard Medium Hard	Medium (10.5- 11.5 %)	Medium	Noodles, steamed breads, hearth breads, flatbreads
CWSWS[d] (AC Reed)	White	Soft	Low (10.5 %)	Weak	Noodles, flatbreads, steamed breads, cakes, cookies, pastry, crackers
CWAD[e] (Kyle)	White	Extra hard	High (13.5 %)	Strong	Macaroni, spaghetti

† Canada western red winter and Canada eastern soft white winter not shown.

[a] CWRS, Canada western red spring.

[b] CWES, Canada western extra strong.

[c] CPS, Canada prairie spring.

[d] CWSWS, Canada western soft white spring.

[e] CWAD, Canada western amber durum.

Japanese udon noodles (Zhao *et al.*, 1998). Consequently, promising Asian-Pacific udon noodle markets have directed wheat quality improvements towards the development of Canadian Prairie Spring (CPS) cultivars with flours possessing reduced amylose concentrations (Demeke *et al.*, 1997b).

In common wheat three Wx proteins, Wx-A1 (~ 59 kD), Wx-B1 (~ 58.8 kD), and Wx-D1 (~ 58.9 kD), are encoded by three homoeologous *Wx* loci and are collectively responsible for synthesizing the amylose component of endosperm starch (Fujita and Taira, 1998; Nakamura *et al.*, 1998; Murai *et al.*, 1999). The wildtype alleles of the A, B and D genomes of wheat are designated *Wx-A1a*, *Wx-B1a*, and *Wx-D1a*, respectively, whereas null alleles that produce no detectable Wx protein are designated *Wx-A1b*, *Wx-B1b*, and *Wx-D1b* (Yamamori *et al.*, 1994). Null mutations are known as mutations that reduce the function of a gene to zero (Russell, 1992). The starch properties important for the eating quality and processing of udon noodles include low amylose, higher starch pasting characteristics, and swelling properties (Oda *et al.*, 1980; Miura and Tanii, 1994). These starch properties have been identified in unadapted partially Wx (reduced amylose wheats with one or two null *Wx* alleles) accessions or cultivars (Yamamori *et al.*, 1994; Demeke *et al.*, 1997b; Graybosch *et al.*, 1998; Zhao and Sharp, 1998). All current Australian soft wheat cultivars accepted for the udon noodle market have a null mutation in the *Wx-B1b* allele (Panozzo and Eagles, 1998). Currently, locally adapted partially waxy cultivars of Canadian Prairie Spring wheat are being developed through the introgression of null *Wx* alleles (*Wx-A1b, Wx-B1b, and Wx-D1b*) (Demeke *et al.*, 1997b; 1999).

Waxy starches are one of the most common specialty starches and have been identified in various crops such as maize (Echt and Schwartz, 1981), rice (*Oryza sativa* L.) (Sano, 1984; Sano *et al.*, 1985), potato (*Solanum tuberosum* L.) (Jacobsen *et al.*, 1989), and barley (*Hordeum vulgare* L.) (Hylton *et al.*, 1996). In contrast to the one *Wx* locus in diploid plants, hexaploid wheat has three *Wx* loci, that is, one *Wx* locus (*Wx-A1*, *Wx-B1*, and *Wx-D1*) in each genome. A naturally occurring fully Wx hexaploid wheat mutant has yet to be identified, possibly because of the complex nature of the genome. That is, the probability of wildtype alleles *Wx-A1a*, *Wx-B1a*, and *Wx-D1a* mutating simultaneously into null alleles *Wx-A1b*, *Wx-B1b*, and *Wx-D1b* and then recombining to produce a spontaneous Wx line is expected to be very low. In Japan, waxy common wheat (amylose-free wheat with three

null *Wx* alleles) has been produced by crossing Kanto 107, a Japanese wheat carrying both *Wx-A1b* and *Wx-B1b* null alleles, with Bai-Huo, a Chinese wheat carrying a *Wx-D1b* null allele (Yamammori *et al.*, 1994). Kanto 107 has been identified by researchers as a promising breeding line with its low amylose content in flour starch and superior texture of udon noodles compared to cultivars with normal amylose content (Hoshino *et al.*, 1985). Until recently, the Chinese landrace Bai-Huo was the only known mutation source carrying a *Wx-D1b* null allele (Kiribuchi-Otobe *et al.*, 1997; Yasui *et al.*, 1997). Partially waxy wheat cultivars are expected to be suitable for the production of high-quality noodles. Waxy wheat may ultimately be useful for waxy starch production where formerly only waxy maize (*Zea mays* L.) has been utilized for these purposes including thickeners, soup mixes, cookies, and syrup (Yasui *et al.*, 1996; Demeke *et al.*, 1997b; Kiribuchi-Otobe *et al.*, 1997; Graybosch *et al.*, 1998).

Recently, researchers have produced double haploid lines of all eight homozygous genotypes (Type 1, *Wx-A1a, Wx-B1a, Wx-D1a*; Type 2, *Wx-A1b, Wx-B1a, Wx-D1a*; Type 3, *Wx-A1a, Wx-B1b, Wx-D1a*; Type 4, *Wx-A1a, Wx-B1a, Wx-D1b*; Type 5, *Wx-A1a, Wx-B1b, Wx-D1b*; Type 6, *Wx-A1b, Wx-B1a, Wx-D1b*; Type 7, *Wx-A1b, Wx-B1b, Wx-D1a*; and Type 8, *Wx-A1b, Wx-B1b, Wx-D1b*) at *Wx* loci in common wheat. These eight genotypes represent a useful resource for the elucidation of the relationships among *Wx* loci null allele genotypes, Wx protein levels, starch chemical and physical properties, and noodle production (Zhao and Sharp, 1998). The availability of these genotypes in different backgrounds will facilitate an extensive characterization in many areas including the molecular characterization null *Wx* alleles in hexaploid wheat. Unlike Wx mutations in rice (Wang *et al.*, 1995), maize (Echt and Schwartz, 1981; Shure *et al.*, 1983; Klosgen *et al.*, 1986; White *et al.*, 1994), potato (van der Leij *et al.*, 1991a), and barley (Domon, 1996), the null mutations in the *Wx* alleles of wheat have not been extensively characterized. Vrinten *et al.* (1999) have proposed that the absent Wx-B1 protein in a waxy wheat line derived by crossing Kanto 107 and Bai-Huo results from the deletion of the *Wx-B1* gene and/or an arm of chromosome 4AL. Their characterization of a 117 bp deletion at the 5' end of the *Wx-A1b* null allele and a 588 bp deletion at the 3' end of the *Wx-D1b* null allele did not account for absent Wx-A1 or Wx-D1 proteins, respectively. Demeke *et al.* (1999) have produced a waxy hexaploid line denoted, CDC Wx2 (type 8), and a partially waxy hexaploid line denoted, CDC Wx6 (type 5) by crossing

Kanto 107 (type 7) and Bai-Huo (type 4). These lines were the basis of the following study of *Wx* transcripts encoded by the null *Wx* genes of hexaploid wheat. This information will contribute to a better understanding of the diversity or redundancy among the *Wx* alleles of hexaploid wheat. The major objectives of the thesis were as follows:

- To isolate and characterize a wheat cDNA encoding a Waxy protein.

- To identify aberrant *Wx* transcripts encoded by the null *Wx-A1b* allele of CDC Wx2, a waxy hexaploid wheat line, which result in an absent Wx-A1 protein (~59 kD).

2.0 LITERATURE REVIEW

2.1 Starch Granule Structure

Starch, a food reserve in plants, is stored in various storage organs such as in the endosperm of cereals, tubers of potato (*Solanum tuberosum*), and roots of cassava (*Manihot esculenta*) (Preiss and Sivak, 1996, 1998). Most of the starch utilized world-wide comes from a relatively small number of crops, the most important being maize, potato, wheat, and tapioca with smaller amounts from rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), sweet potato (*Ipomoea batatas*), arrowroot (*Maranta arundinacea*), sago (*Metroxylon sagu*; *M. rumphii*), and mung beans (*Phaseolus vulgaris*) (Wang *et al.*, 1998). In general, starches from tapioca and sorghum are used only for foods whereas those from maize, potato, and wheat are used for both food and non-food purposes. Starch consists of a mixture of two types of glucose polymers including a relatively unbranched amylose and a highly branched amylopectin fraction (Takeda *et al.*, 1986). In the endosperm and roots of plants, starch is synthesize and stored as starch granules within specialized colorless plastids known as amyloplasts. Cereal endosperm starches are composed of ~ 25 % amylose and ~ 75 % amylopectin, although, as discussed in detail in later sections, mutations affecting starch biosynthesis can significantly affect the amount of amylose molecules in the starch granule.

Starch granules vary widely in shape and size among species and organs (Hoseney, 1986). In wheat, starch granules (~ 1 μm in diameter) are present in the developing endosperm within a few days after anthesis and occupy a minute fraction of the amyloplast stromal volume (Stark and Lynn, 1992; Rahman *et al.*, 1995). By maturity, starch deposition has proceeded to the point where starch granules occupy essentially the entire amyloplast stroma. Mature wheat has a bimodal distribution of starch granules composed of a lenticular shaped 'A' granule population (10-30 μm in diameter) and a 'B' granule population of spherical granules (diameter < 10 μm). While the 'B' granules are numerous, constituting > 95 % of the total granule number, the 'A' granule population contains 70-80 % of starch mass. 'A' granules are initiated in the early cell division phase of endosperm

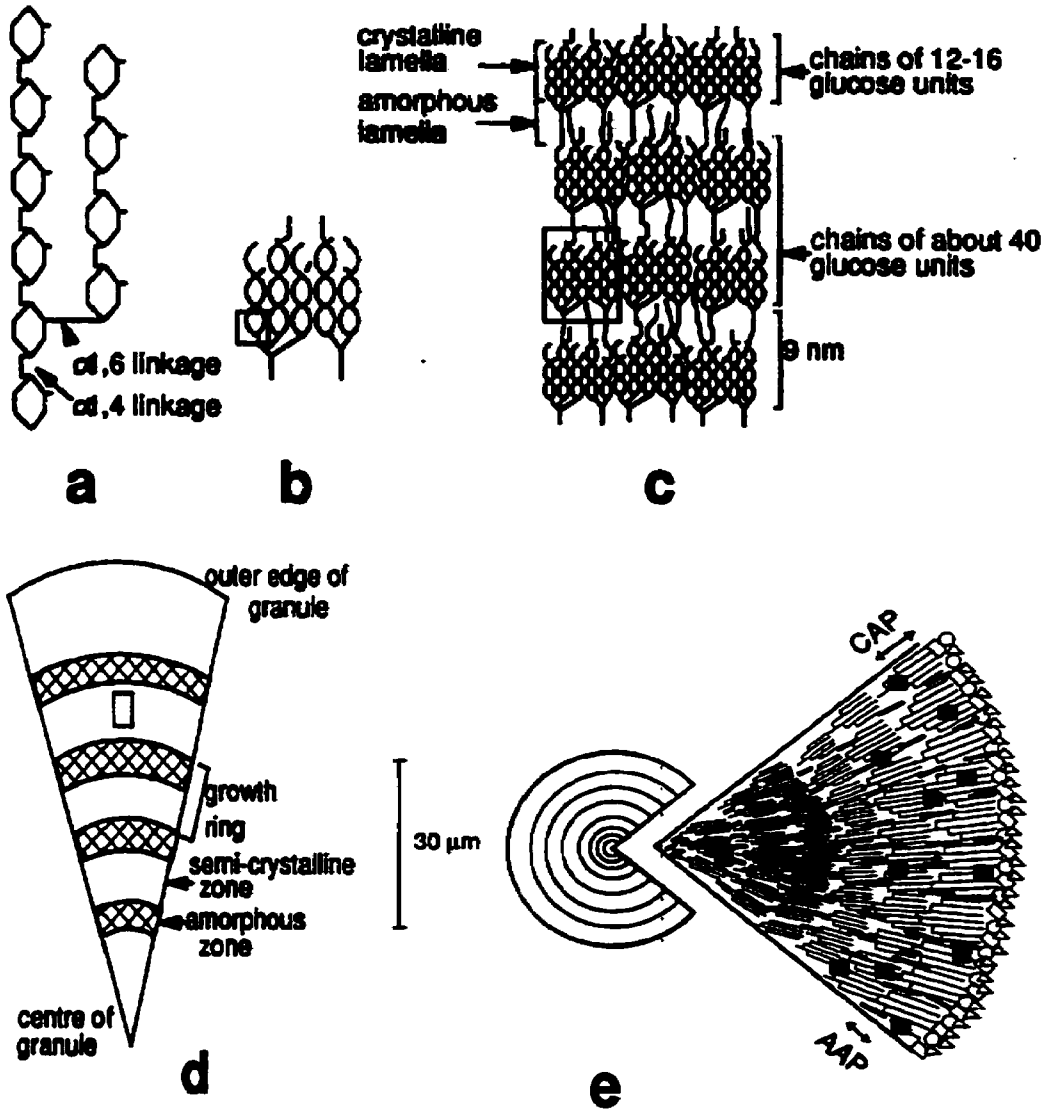
development, while 'B' granule initiation begins ~ 16 days post anthesis (DPA) and continues throughout the cell expansion phase (Morrell *et al.*, 1995). Starch granules exert a minimal effect on the osmotic pressure of the cell. Kernel desiccation leads to the disruption of amyloplast membranes and the exposure of the starch granules to the protein matrix of the endosperm (Hoseney, 1986). The starch granules isolated from the mature grain contain two types of proteins: the proteins embedded within the starch granule during starch deposition in the amyloplast and the proteins exclusively associated with the surface of the granule, that have come in contact with the starch granule during maturation or isolation of starch granules (Schofield and Greenwell, 1987).

Glucose is the basic building block of both amylose and amylopectin. Alpha-amylose is an essentially linear polymer made up of several thousands of α-1,4-linked glucose residues with a molecular weight between 5×10^5 to 10^6 (Takeda *et al.*, 1986). In contrast, amylopectin has a molecular weight of several millions and is a highly branched glucose polymer consisting mainly of α-1,4 linked glucose residues with α-1,6 branch points (2-4 %; Fig. 2.1a). Amylose is not essential to the formation of the granule. The basic structure of the granule is dictated by the packing of amylopectin molecules in organized arrays (Smith *et al.*, 1997). The chains within the granule are radially arranged with their non-reducing ends pointing toward the surface and are organized into alternating crystalline and amorphous lamellae with a periodicity of 9 nm. The lamellae reflect the arrangement of chains into clusters. Within clusters, chains associate to form double helicies that pack together in ordered arrays to give the crystalline lamellae. The amorphous lamellae contain the branch points (Fig. 2.1b,c). Regions of alternating crystalline and amorphous lamellae form concentric zones within the granules (~ hundreds of nm in width). These semicrystalline zones alternate with amorphous zones. A semicrystalline-amorphous repeat is referred to as a growth ring (Fig. 2.1d,e). Within this basic outline of the organization within a starch granule there is a large amount of genetically, developmentally, and environmentally induced variation.

2.2 Starch Synthesis

A generalized pathway for the production of starch will be presented herein. Intricate details of the pathway and the enzymes involved have been extensively reviewed

Fig. 2.1 Schematic representation of levels of organization within a starch granule. The boxes within the diagrams in panels b, c, and d represent the area occupied by the structure in the preceding panel. (a) Structure of two branches of an amylopectin molecule, showing individual glucose units. (b) A single cluster within an amylopectin lamellae. (c) The crystalline lamellae are produced by the packing of double helices in ordered arrays. Chains of 12-16 glucose units span one cluster. Chains of about 40 glucose units span two clusters. (d) Slice through a granule showing alternating zones of semi-crystalline material, consisting of crystalline and amorphous lamellae, and amorphous material. Adapted from Smith *et al.* (1997). (e) Slice through a granule (30 μm diameter), showing crystalline amylopectin (CAP), amorphous amylopectin (AAP), amylose molecules (thick unbranched lines), protein molecules associated with the surface (open symbols), and proteins located within the granule matrix (filled symbols). Adapted from Morrel *et al.* (1995).


α1,6 linkage
α1,4 linkage
a
b
crystalline lamella
amorphous lamella
chains of 12-16 glucose units
chains of about 40 glucose units
9 nm
c
outer edge of granule
growth ring
semi-crystalline zone
amorphous zone
centre of granule
d
30 µm
CAP
AAP
e

by other reports (Martin and Smith, 1995; Nelson and Pan, 1995; Morrell *et al.*, 1995; Preiss and Sivak, 1996, 1998; Koβmann *et al.*, 1997; Smith *et al.*, 1997; Wang *et al.*, 1998). The final steps of starch biosynthesis (Fig. 2.2) involve three enzymes including ADP-glucose pyrophosphorylase (ADP-GPPase; EC 2.7.7.23), starch synthases (granule-bound starch synthase isoforms I and II [GBSS] and soluble starch synthase isoforms I, II, and III [SSS]; EC 2.4.1.21), and starch branching enzyme isoforms I and II (BE or SBE; EC 2.4.1.18) (Martin and Smith, 1995). ADP-GPPase catalyzes the formation of ADP-Glucose, the basic building block of amylose and amylopectin, from α-D-glucose-1-phosphate (G1P) as follows:

α-D-glucose-1-phosphate + ATP ↔ ADP-Glucose + pyrophosphate (PPi)

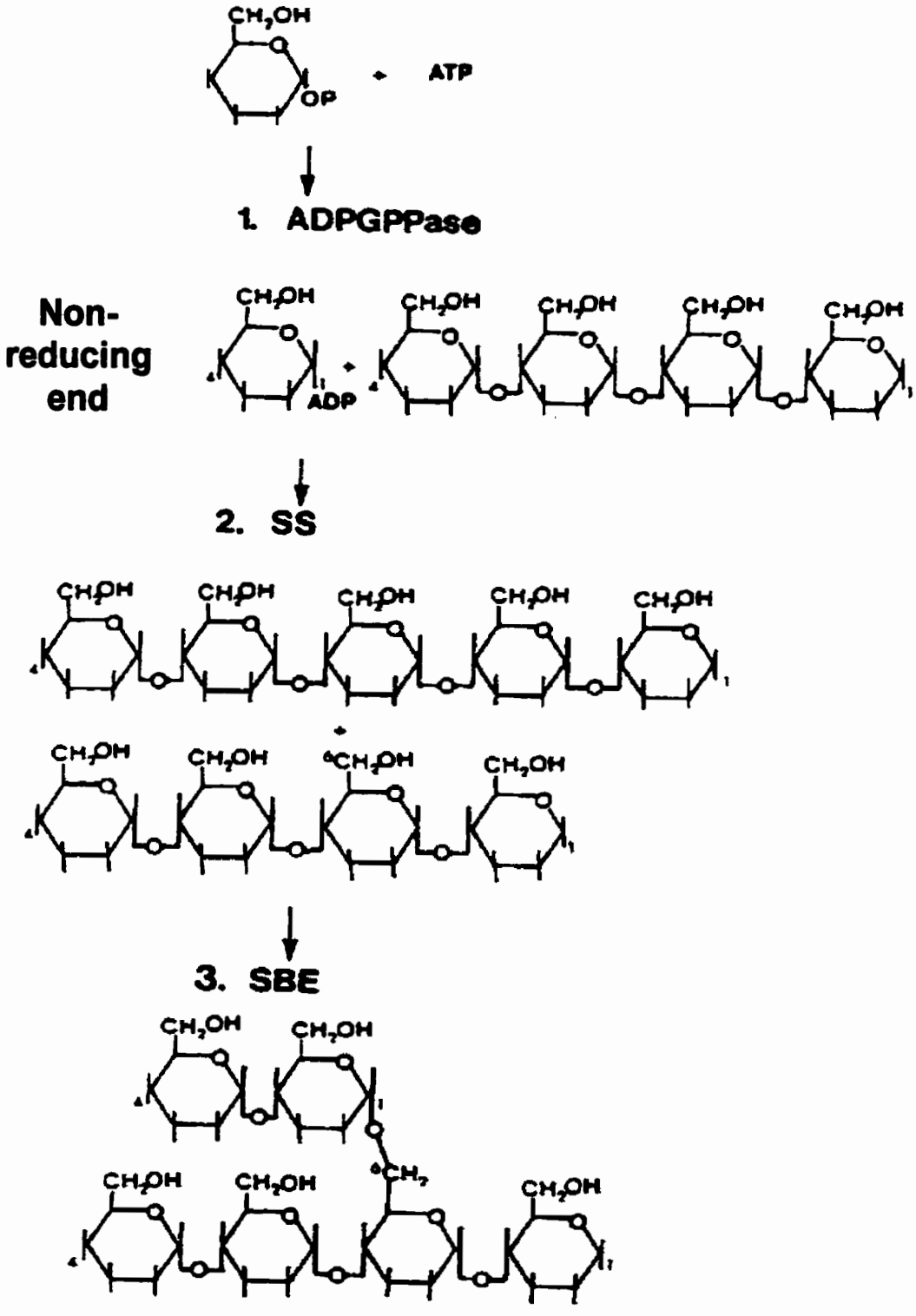
The first enzyme is responsible for the synthesis of the substrate in all plants (Smith *et al.*, 1997) and the other two for the production of amylose and amylopectin polymers. ADP-GPPase is considered by many to be the key enzyme in the regulation of the pathway because it is allosterically regulated by both inorganic phosphate (an inhibitor) and 3-phosphoglycerate (an activator) (Preiss and Sivak, 1996). Starch synthases (SS) form α-1,4 linkages between the non-reducing end of a glucose polymer and ADP-glucose molecules as follows:

$(\{1,4\}\text{-}\alpha\text{-D-glycosyl})_n + \text{ADP-Glucose} \rightarrow (\{1,4\}\text{-}\alpha\text{-D-glycosyl})_{n+1} + \text{ADP}$

In vivo, it is uncertain whether all SS use both amylose and amylopectin as substrates. Lastly, BE catalyzes the formation of the α-1,6 linkage found in starch polymers. Branched chains of α-glucans are produced from linear glycosyl chains of α-glucan through the formation of α-1,6 linkage branch points. There are a number of different isoforms of each enzyme, some of which show differences in organ specificity and temporal regulation. The contribution of the different isoforms to the overall process of starch biosynthesis in most tissues is not known.

Debranching enzymes, isoamylases and pullulanases, (DBE; EC 3.2.1.41 and EC 3.2.1.68) hydrolyze α-(1,6)-glucose linkages and are also considered to play a role in the synthesis of starch (James *et al.*, 1995; Ball *et al.*, 1996). Debranching enzyme-deficient mutants of maize, rice, and *Chlamydomonas* have been isolated and all accumulate

Fig. 2.2 Steps of starch biosynthesis. ADP-GPPase catalyzes the formation of ADP-glucose and inorganic pyrophosphate from glucose-1-phosphate and ATP (step 1). Starch synthases (SS) add glucose units from ADP-glucose to the non-reducing end of a growing α-(1,4)-linked glucan chain by an α-(1,4)-linkage and release ADP (step 2). Two enzymatic functions are associated with starch-branching enzymes (SBE), namely cleavage of α-(1,4)-glycosidic linkages on the glucan polymer and reattachment of the released chain through an α-(1,6)-linkage to the same or another α-(1,4)-linked glucan chain (step 3). Adapted from Martin and Smith (1995).


ATP
1. ADPGPPase
Non-reducing end
ADP
2. SS
3. SBE

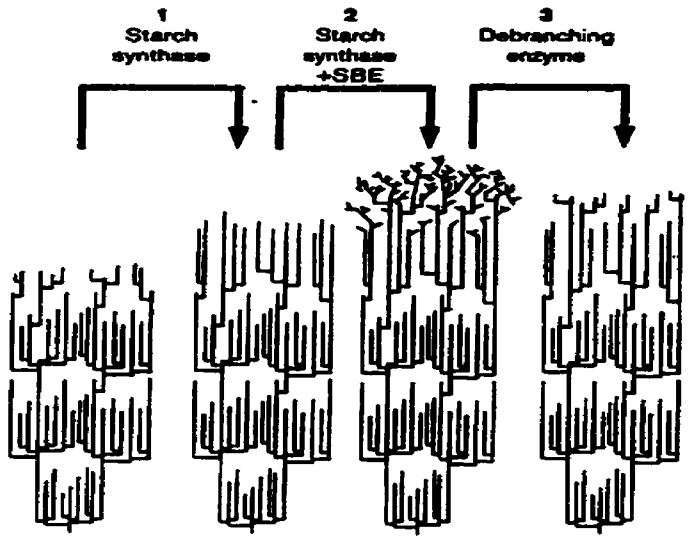
phytoglycogen, a glucose polymer, which is more similar to animal glycogen than to plant starch in that it posesses a greater proportion of α-(1,6)-linkages and is water soluble. The action of debranching enzymes has been suggested to be important in 'pre-amylopectin trimming' (Fig. 2.3a). The pre-amylopectin trimming model proposes a direct involvement of DBE in amylopectin synthesis (Ball *et al.*, 1996; Mouille *et al.*, 1996; Smith *et al.*, 1999). It is speculated that a sequence of synthetic events at the surface of the granule creates a cluster within an amylopectin molecule, as follows: (1) short chains are elongated by starch synthase; (2) when chains reach a sufficient length to become substrates for SBE, a highly branched pre-amylopectin is formed; (3) selective trimming of this structure by DBE creates a bed of short chains from which the next round of synthesis can occur. When DBE activity is reduced or eliminated, pre-amylopectin accumulates. The production of phytoglycogen has been detected in mutants such as *su1*, *sta7*, and *dbe1*. Mouille *et al.* (1996) have suggested that the production of pre-amylopectin is a mandatory step for the production of starch; however, proof that the trimming mechanism actually operates *in vivo* is still lacking.

Another explanation for the accumulation of phytoglycogen in DBE-deficient cells has led researchers to propose that DBE is not directly involved in amylopectin synthesis (Zeeman *et al.*, 1998). The soluble glucan recycling model (Fig. 2.3b) proposes that DBE is only indirectly involved in starch synthesis. Amylopectin synthesis requires only SS and SBE. In a normal plastid (Fig. 2.3b, top), small soluble malto-oligosaccharides in the stroma may be elongated by SS, and then branched by SBE. Any glucans thus synthesized will be degraded by a suite of enzymes including DBE (dashed lines), preventing the accumulation of such products. In plastids in which DBE activity is reduced or eliminated (Fig. 2.3b, bottom), this degradative mechanism is incomplete. Soluble branched glucans formed by SS and SBE from malto-oligosaccharides can be further elaborated, providing more substrates for SS and SBE, and leading to both an accumulation of phytoglycogen and a reduction in the rate of starch synthesis. At present the question of whether DBE is, directly or indirectly, involved in amylopectin synthesis remains open for discussion.

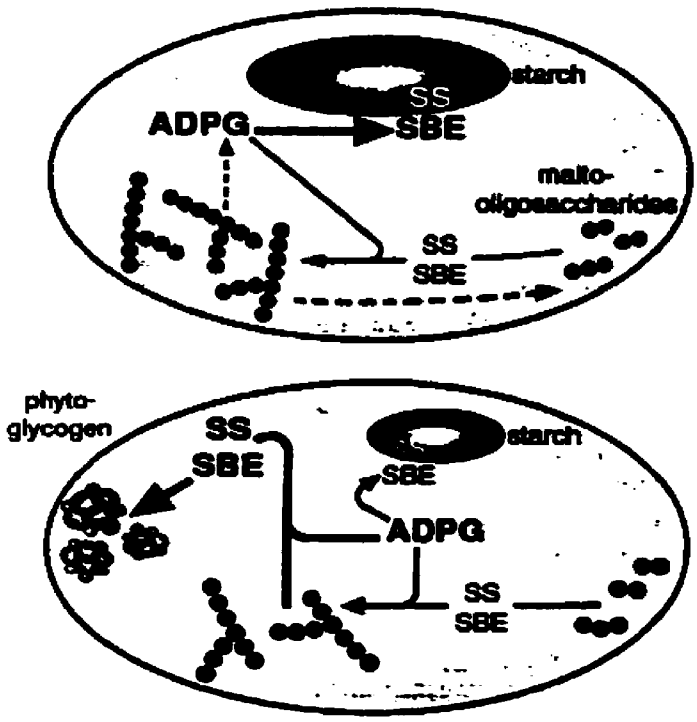
In wheat, barley, and rice, sucrose is transported from leaves through the phloem vascular tissue and unloaded at the developing endosperm (Singh and Metha, 1986; Fig. 2.4). In leaves, starch is deposited in granules in the chloroplasts during active carbon

Fig. 2.3 Models to explain the involvement of debranching enzyme in starch synthesis. (a) Pre-amylopectin trimming model. (b) The soluble glucan recycling model. Adapted from Smith *et al.* (1999).

(a)


1
Starch
synthase
2
Starch
synthase
+SBE
3
Debranching
enzyme

(b)


starch
SS
ADPG
SBE
malto-
oligosaccharides
SS
SBE
phyto-
glycogen
SS
SBE
starch
SBE
ADPG
SS
SBE

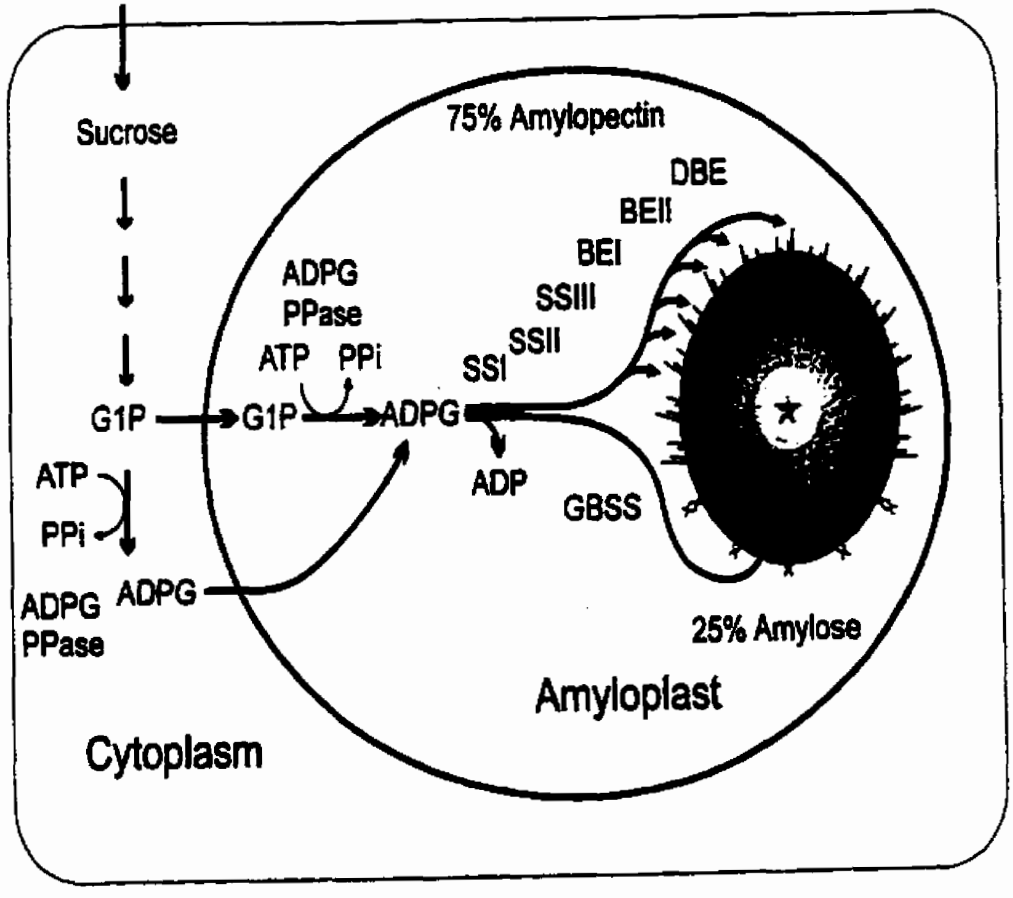


Fig. 2.4 General scheme for starch biosynthesis in cereals. Glucose-1-phosphate (G1P), ADP-glucose pyrophosphorylase (ADP-GPPase), ADPG (ADP-glucose), starch synthase isoforms (SSI, SSII, and SSIII), granule-bound starch synthase (GBSS), starch branching enzyme isoforms (BEI and BEII), and debranching enzyme (DBE). Adapted from Rahman *et al.* (1999).

dioxide fixation by photosynthesis during the day and degraded by respiration during darkness (Preiss and Sivak, 1996). The accumulated starch is required for sucrose synthesis at night and subsequently transported from the leaf to sink tissues. Starch remobilization ensures continuous availability of photosynthates to the whole plant. The biosynthesis and degradation of leaf starch is more dynamic than the metabolism in reserve tissues. Sucrose is converted to G1P through the action of invertase, sucrose synthase, UDP-glucose pyrophosphorylase, hexokinases, and phosphoglucomutase. The supply of substrate to ADP-GPPase differs in different tissues and organisms. It is generally accepted that in storage tissues such as the endosperm or cotyledons, hexose phosphates are imported into the amyloplast whereas fructose-6-phosphate from the reductive pentose phosphate pathway is utilized in photosynthetic plastids (Keeling *et al.*, 1988; Bowsher *et al.*, 1996). In cereals, there have been clear demonstrations that G1P is the imported substrate rather than ADP-glucose or glucose-6-phosphate. There is evidence from barley and maize that most ADP-GPPase activity is located outside the plastid and that ADP-glucose is specifically transported into the plastid by a protein (Kleczkowski, 1996).

The general scheme outlined in Fig. 2.4 is based on studies of starch biosynthesis in species such as maize, pea (*Pisum sativum*), potato, and *Chlamydomonas* (Ball *et al.*, 1996; Martin and Smith, 1995; Smith *et al.*, 1997; Nelson and Pan, 1995). The basic principles of starch synthesis in these storage organs are expected to be similar in wheat endosperm; however, future research will likely identify significant differences in detail. In cereals, there appear to be at least four classes of SS important to starch synthesis in the endosperm: GBSS (Shure *et al.*, 1983), SSI (Knight *et al.*, 1998), SSII (Harn *et al.*, 1998), and SSIII (Gao *et al.*, 1998). GBSS is essential for amylose synthesis, and may also be a contributor to amylopectin synthesis. The roles of SSI, SSII, and SSIII are thought to be predominantly in amylopectin synthesis although they may be non-essential contributors to the synthesis of amylose (Craig *et al.*, 1998; Gao *et al.*, 1998). Two classes of BE are known as BEI and BEII. The BEII class in maize contains two members, BEIIa and BEIIb (Boyer and Preiss, 1978). Currently, it is thought that BEII, instead of BEI, especially BEIIb may have the most influence on amylopectin branching (Boyer and Preiss, 1978; Boyer *et al.*, 1980; Bhattacharyya *et al.*, 1990; Mizuno *et al.*, 1993). As detailed above, the role of DE in starch synthesis is the subject of ongoing discussion. Our understanding of starch biosynthetic

enzymes (Preiss and Sivak, 1996) has outstripped our understanding of the nature and regulation of the starch biosynthetic process. Considerable research will be needed to fully understand the highly integrated and regulated process of amylopectin synthesis and its organization to form a starch granule.

2.3 Bacterial Glycogen

Many bacteria can accumulate glycogen as an energy reserve during growth or at the end of the growth phase (Preiss and Romeo, 1989). Glycogen is a branched glucose polymer consisting of ~ 90 % α-(1,4)-linkages and the rest in α-(1,6)-linkages. Glycogen usually accumulates in the bacterial cell when there is excess carbon in the media and when growth is limited by a lack of required nutrient for growth. Glycogen can accumulate to >50% of dry cell weight; however, levels of accumulation depend on both the nutritional content of the media and the growth phase of the organism. The high molecular weight and physical properties of stored glycogen, acculumated to low or high levels, has litlle effect on the internal osmotic pressure of the cell.

In 1964, it was demonstrated that bacteria possess both ADP-GPPase (Shen and Preiss, 1964) and ADP-glucose specific glycogen synthase (Greenberg and Preiss, 1964). ADP-GPPase converts ATP and G1P into ADP-glucose and pyrophosphate. Glycogen synthase converts ADP-glucose and α-glucan into α-1,4-glucosyl-glucan and ADP. Subsequently, it has been shown that branching enzyme activity was also present in many bacterial extracts (Holmes *et al.*, 1982). Thus, glycogen accumulating bacteria have the enzymes of the ADP-glucose pathway to synthesize α-1,4-glucosidic linkages, as well as BE activity for synthesis of the α-1,6-glucosidic linkages of glycogen.

It is important to note that glycogen is not required for growth since mutants of *E. coli*, including deletion mutants (Govons *et al.*, 1969) which have defective structural genes for glycogen biosynthetic enzymes (e.g, glycogen synthase), and are therefore unable to synthesize glycogen, grow as well as their normal parental strains (Preiss and Romeo, 1989). These energy reserves are degraded and utilized as a source of carbon that is no longer available from the media or environment. The function of glycogen, an endogenous source of carbon, within bacterial cells remains unclear; however, it is thought to be important in the prolongation of cell survival and in the formation and maturation of spores.

2.4 Polyploid Nature of the Wheat Genome

Wheat (*Triticum* L.), belonging to the grass family Gramineae, subfamily Festucoideae, and tribe Festuceae, is thought to have originated in the fertile crescent, encompassing Turkey, Syria, Iraq, and Iran (Williams, 1993). It was distributed to areas such as West Asia, Ethiopia and the north-eastern countries of Africa, and Turkey and southern Russia, which became centres of diversification for diploid, tetraploid, and hexaploid wheats, respectively. In contrast, *T. spelta*, a hexaploid species, had its centre of diversification in the upper Rhine area of Germany. Two species, *T. boeoticum* Boiss. em. Schiem (Wild einkorn) and *T. monococcum* L. (Einkorn) are known as diploid wheats (2n=2x=14 chromosomes). Eight species, *T. dicoccoides* Korn. (Wild emmer), *T. timopheevi* Zhukov. (Timopheevi wheat), *T. dicoccum* Schubl. (Emmer wheat), *T. durum* Desf. (Durum wheat), *T. turgidum* L. (Rivet wheat), *T. turanicum* Jakubz. (Khorasan wheat), *T. polonicum* L. (Polish wheat), and *T. carthlicum* Nevski (Persian wheat) belong to the tetraploid wheats (2n=4x=28 chromosomes). Six subspecies, *T. aestivum* L. em. Thell subsp. *spelta* (L) Thell (Spelt wheat), *T. aestivum* subsp. *macha* (Macha wheat), *T. aestivum* subsp. *vavilovi* (Tuman) Sears n. comb., *T. aestivum* subsp. *vulgare* (Vill. Host) Mackey (Common wheat), *T. aestivum* subsp. *compactum* (Host Mackey), and *T. aestivum* subsp. *sphaerococcum* (Perc. Mackey) (Shot wheat), comprise the hexaploid group of wheats (2n=6x=42 chromosomes).

The classical example of alloploidy, plants derived from the combination of genomes that are different, in crop plants is provided by wheat (Briggs and Knowles, 1967). Diploid, tetraploid, and hexaploid wheats have two, four, and six 7-chromosome sets or one, two and three genomes, respectively (Williams, 1993). Tetraploid wheats (AABB) are believed to have originated from a natural cross between a variety of the species *T. boeoticum* known as *T. urartu* (AA), which contributed the A genome and a diploid donor of the B genome. The exact donor of the B genome remains unclear. Hexaploid wheats (AABBDD), believed to have been derived from a natural cross, are composed of the A and B genomes in tetraploid wheats and the D genome is contributed by *Triticum tauschii* (syn. *Aegilops squarrosa*). Each genome consists of seven pairs of homologous chromosomes (Fig. 2.5). Each chromosome pair is genetically similar to one specific chromosome pair of each of the two remaining genomes. Wheat chromosomes are divisible into seven

homoeologous groups. The location and structure of genes on each member of these homoeologous groups is virtually identical. Durum wheats are allotetraploid and contain only the A and B genomes. The identification of mutants in allopolyploids is hindered by the ploidy of their genomes, that is, where more than one genome is present, gene mutations often fail to manifest themselves because genes in other genomes will carry on their function (Briggs and Knowles, 1967).

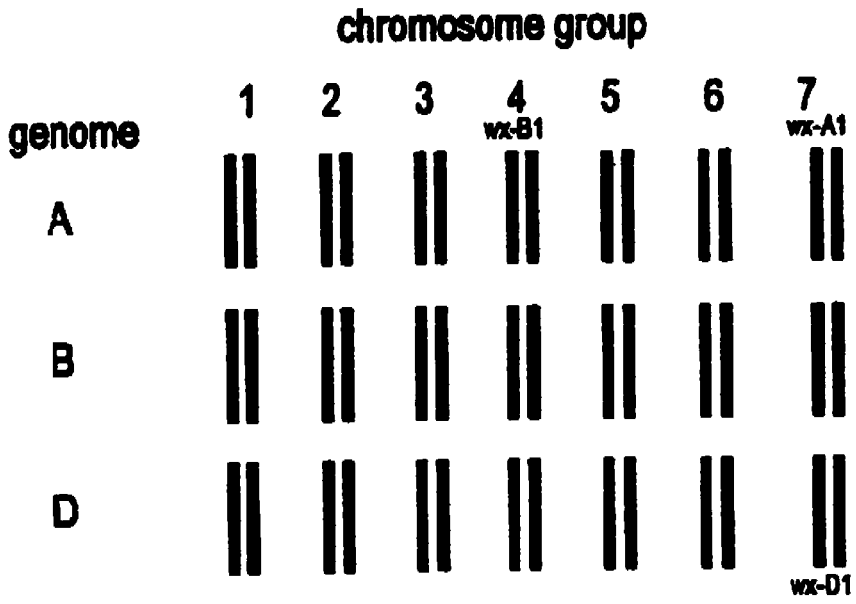


Fig. 2.5 Organization of common wheat chromosomes (2n=6x=42 chromosomes) and location of *Wx* loci. Adapted from Graybosch (1998).

2.5 Waxy Wheat Mutants

The Wx protein, also known as GBSSI, encoded by the *Wx* locus is the key enzyme responsible for synthesizing amylose polymers in the amyloplasts of plants (Martin and Smith, 1995). Currently, in all species that have been investigated the mature ~59 kD Wx protein is found embedded within starch granules (Nelson *et al.*, 1978; Echt and Schwartz, 1981). The Wx protein is found exclusively within or near the surface of the granules and is absent from the soluble phase of amyloplasts. The ~59 kD Wx protein (Yamamori *et al.*, 1992) in the endosperm starch of common hexaploid wheat has been separated into three isoforms using two-dimensional SDS-PAGE (Nakamura *et al.*, 1995a). These three Wx proteins, Wx-A1, Wx-B1, and Wx-D1, have slightly different molecular weights (Murai *et al.*, 1999) and/or isoelectric points (pI), ranging from 5.5 to 6.5 (Nakamura *et al.*, 1995a). Zhao and Sharp (1996) developed a 1D-SDS-PAGE system, replacing the cumbersome 2D-SDS-PAGE procedure, that separates the Wx-A1, Wx-B1, and Wx-D1 proteins in hexaploid wheat using only the distal half of a single mature grain. Using nulli-tetrasomic lines of Chinese Spring wheat the chromosomal location of the three *Wx* loci have been identified (Chao *et al.*, 1989; Ainsworth *et al.*, 1993; Nakamura *et al.*, 1995b; Fig. 2.5). The Wx-A1 protein is encoded by the *Wx-A1* gene on the short arm of chromosome 7A. The Wx-B1 protein is encoded by the *Wx-B1* gene on the long arm of chromosome 4A (translocated from 7BS). Lastly, the Wx-D1 protein is encoded by the *Wx-D1* gene on the short arm of chromosome 7D of wheat. Recently, genomic DNA sequences encoding Wx-A1, Wx-B1, and Wx-D1 proteins in hexaploid wheat have been isolated and characterized (Murai *et al.*, 1999). Deduced molecular weights of 59.0 kD for a Wx-A1 protein, 58.8 kD for a Wx-B1 protein, and 58.9 kD for a Wx-D1 protein were predicted from the isolated *Wx* genes. Ainsworth *et al.* (1993) have characterized the expression of *Wx* genes in wheat. Developing grains (5, 10, 15, 20, and 25 DPA) of Chinese spring wheat accumulated a 2.4 kb *Wx* mRNA transcript throughout grain filling with peak expression of the *Wx* genes at ~10 to 20 DPA.

The nucleotide sequence of one full length wheat complementary DNA (cDNA) clone encoding a Wx-A1 protein has been isolated (Accession number X57233; Clark *et al.*, 1991). The cDNA sequence (2186 nucleotides in length) has an open reading frame of 1845 nucleotides, a 5' untranslated region of 63 nucleotides, a 3' untranslated region of 278

nucleotides, and a poly(A) tail of 18 residues. The deduced protein contains 615 amino acids with a calculated molecular weight of 67.8 kD; however, this protein includes a 7.7 kD transit peptide which likely targets the Wx protein to plastids (Klosgen *et al.*, 1989; Klosgen and Weil, 1991). None of the three Wx peptide amino acid sequences reported by Murai *et al.*, (1999) contained an 11 amino acid insertion, first described by Clark *et al.* (1991), that is thought to be unique to wheat (Ainsworth *et al.*, 1993). cDNA clones encoding for the Wx protein have been isolated from maize (Shure *et al.*, 1983; Klosgen *et al.*, 1986), rice (Okagaki, 1992), cassava (Salehuzzaman *et al.*, 1993), and potato (Visser *et al.*, 1989). These Wx cDNA clones have subsequently been used to study 1) biological activity, expression, and regulation of *Wx* genes *in planta* (Visser *et al.*, 1991a,b; van der Leij *et al.*, 1991a; van der Steege *et al.*, 1992; Flipse *et al.*, 1996a), 2) co-supression of endogenous *Wx* genes (Flipse *et al.*, 1996b), 3) antisense inhibition of the *Wx* transcript using homologous (Visser *et al.*, 1991b; Shimada *et al.*, 1993; Kuipers *et al.*, 1994b) or heterologous *Wx* genes (Salehuzzaman *et al.*, 1993), 4) inheritance and segregation of *Wx* transgenes (Flipse *et al.*, 1996a), and 5) formation and deposition of amylose in transgenic potato tubers expressing a *Wx* gene (Kuipers *et al.*, 1994a).

In plants, Wx mutants are characterized by both the lack of Wx proteins (i.e., null alleles) and amylose (Sprague *et al.*, 1943; Nelson and Rines, 1962; Tsai, 1974; Okuno and Sakaguchi, 1982; Sano, 1984; Hseih, 1988; Jacobsen *et al.*, 1989; Hylton *et al.*, 1996), with the exception of several maize Wx mutants which possessed a non-functional Wx protein (Echt and Schwarz, 1981). In contrast, non-Wx phenotypes are characterized by the presence of functional Wx proteins and the production of ~ 25-30 % amylose (Hoseney, 1986). The absence of a Wx protein in amylose-free mutants has led researchers to assume that the Wx protein is responsible for synthesizing amylose in potato (Hovenkamp-Hermelink *et al*, 1987; Visser *et al.*, 1991b; van der Leij *et al.*, 1991a), wheat (Nakamura *et al.*, 1993b; Yamamori and Nakamura, 1994), rice (Sano *et al.*, 1985; Shimada *et al.*, 1993), and maize (Imam, 1989). The best evidence that the Wx protein is responsible for amylose synthesis has been offered by antisense inhibition of the *Wx* transcript in potato tubers which eliminated the production of both amylose and the Wx protein (Visser *et al.*, 1991b; Kuipers *et al.*, 1994b, 1995; Salehuzzaman *et al.*, 1993) and by transformation of an amylose-free

potato mutant with a *Wx* gene resulting in the restoration of amylose synthesis (van der Leij *et al.*, 1991a; Flipse *et al.*, 1994).

Mutations at the *Wx* locus do not appear to affect other tissue specific isoforms of GBSS in wheat (Fujita and Taira, 1998). The Wx protein is considered to be the only SS involved in amylose synthesis in storage organs which permanently store starch. Granule-bound starch synthase isoform II, GBSS II, with a mature molecular weight of 56 kD has recently been identified in the pericarp (Nakamura *et al.*, 1998), aleurone layer, and immature embryos of Wx wheat mutants (Fujita and Taira, 1998). Endosperm and pollen starch granules of Wx wheat mutants lacked both amylose and the 60 kD Wx protein; however pericarp, aleurone, and embryo starch granules contained amylose and a 56 kD protein (Nakamura *et al.*, 1998). Ainsworth *et al.* (1993) also identified leaf-tissue specific expression of a 1.6 kb *Wx* transcript. GBSSII may prove to have an important function in amylose synthesis in non-storage tissues (e.g., photosynthetic tissues, tissues near meristematic tissues, flowers, and fruits) which temporarly store starch. Nucleotide sequences of wheat cDNA clones encoding GBSS II currently remain unpublished.

2.6 Crossing Kanto 107 and Bai-Huo to Produce Fully Waxy Wheat

The production of Wx wheat using various parental combinations has been outlined by various researchers (Yamamori and Nakamura, 1994; Yamamori *et al.*, 1995; Hoshino *et al.*, 1996; Kiribuchi-Otobe *et al.*, 1997, 1998; Zhao and Sharp, 1998). This section discusses the F_2 phenotypes that can be expected when crossing Kanto 107, a double null Wx line, and Bai-Huo, a single null Wx line. A branch diagram derivation of the relative frequencies of the eight phenotypic classes in the F_2 of a cross with three *Wx* genes segregating independently is shown in Fig. 2.6. The wildtype alleles of the A, B, and D genomes are designated *Wx-A1a*, *Wx-B1a*, and *Wx-D1a*, respectively, whereas null alleles that produce no detectable Wx protein are designated *Wx-A1b*, *Wx-B1b*, and *Wx-D1b* (Nakamura *et al.*, 1993a). The independently assorting character pairs in the cross are presence versus absence of a Wx-A1 protein, presence versus absence of a Wx-B1 protein, and presence versus absence of a Wx-D1 protein. There are 64 combinations of eight maternal and eight paternal gametes. (Note: In flowering plants, the union of one

Fig. 2.6. A branch diagram derivation of the relative frequencies of the eight phenotypic classes in the F_2 of a cross with three *Wx* genes segregating independently. Adapted from Russell (1992).

Kanto 107 (Seed parent) **X** **Bai-Huo (Pollen parent)**

(*Wx-A1b/-A1b, Wx-B1b/-B1b, Wx-D1a/-D1a*) (*Wx-A1a/-A1a, Wx-B1a/-B1a, Wx-D1b/-D1b*)

↓

F_1 (*Wx-A1a/-A1b, Wx-B1a/-B1b, Wx-D1a/-D1b*) X F_1 (Selfing)

↓

F2 phenotypes for:			F_2 phenotypic Proportions
Wx-A1a/-A1b	***Wx-B1a/-B1b***	***Wx-D1a/-D1b***	
3/4 *A1a/A1_*	3/4 *B1a/B1_*	3/4 *D1a/D1_*	27/64 *A1a/A1_,B1a/B1_,D1a/D1_*
		1/4 *D1b/D1b*	9/64 *A1a/A1_,B1a/B1_,D1b/D1b*
	1/4 *B1b/B1b*	3/4 *D1a/D1_*	9/64 *A1a/A1_,B1b/B1b,D1a/D1_*
		1/4 *D1b/D1b*	3/64 *A1a/A1_,B1b/B1b,D1b/D1b*
1/4 *A1b/A1b*	3/4 *B1a/B1_*	3/4 *D1a/D1_*	9/64 *A1b/A1b,B1a/B1_,D1a/D1_*
		1/4 *D1b/D1b*	3/64 *A1b/A1b,B1a/B1_,D1b/D1b*
	1/4 *B1b/B1b*	3/4 *D1a/D1_*	3/64 *A1b/A1b,B1b/B1b,D1a/D1_*
		1/4 *D1b/D1b*	1/64 *A1b/A1b,B1b/B1b,D1b/D1b*

F_2 phenotypic proportions	Endosperm starch phenotype using SDS-PAGE †			Endosperm starch phenotype using iodine staining
	Wx-A1	Wx-B1	Wx-D1	
27/64 *A1a/A1_,B1a/B1_,D1a/D1_*	+	+	+	Blue-Black
9/64 *A1a/A1_,B1a/B1_,D1b/D1b*	+	+	-	Blue-Black
9/64 *A1a/A1_,B1b/B1b,D1a/D1_*	+	-	+	Blue-Black
3/64 *A1a/A1_,B1b/B1b,D1b/D1b*	+	-	-	Blue-Black
9/64 *A1b/A1b,B1a/B1_,D1a/D1_*	-	+	+	Blue-Black
3/64 *A1b/A1b,B1a/B1_,D1b/D1b*	-	+	-	Blue-Black
3/64 *A1b/A1b,B1b/B1b,D1a/D1_*	-	-	+	Blue-Black
1/64 *A1b/A1b,B1b/B1b,D1b/D1b*	-	-	-	Reddish-Brown

† **Presence (+) or absence (-) of waxy protein.**

sperm nucleus with the egg nucleus to form the diploid zygote, and of the other sperm nucleus with the two polar nuclei to form a triploid endosperm nucleus [Genotype F1 endosperm tissue: *Wx-A1b/-A1b, Wx-B1b/-B1b, Wx-D1a/-D1a,* two doses from the seed parent, and *Wx-A1a/-A1a, Wx-B1a/-B1a, Wx-D1b/-D1b,* one dose from the pollen parent] is known as double fertilization. The embryo derives from the diploid zygote and the triploid endosperm *Wx-A1b/-A1b/-A1a, Wx-B1b/-B1b/B1a, Wx-D1a/-D1a/-D1b,* stored food within the kernel, derives from the triploid endosperm cell.). Combinations of these haploid F_1 gametes will give rise to 27 different genotypes and eight phenotypes. The eight different endosperm starch phenotypes can be distinguished using 1D-SDS-PAGE. Iodine staining of the embryo-less portion of the kernel can quickly separate fully Wx lines (endosperm starch stains reddish-brown) from non-Wx F_2 lines (endosperm starch stains blue-black).

2.7 Gene Regulation

In eukaryotes, the production of functional proteins involves transcription, a process synthesizing RNA molecules from a DNA template, within the nucleus and translation, a process synthesizing proteins from an mRNA template, within the cytoplasm (Farrell, 1993; Fig. 2.7). The primary RNA transcript, pre-mRNA molecule, undergoes post-transcriptional processing in the nucleus, including the addition of 5' cap, methylation, polyadenylation, and intron splicing, to produce the mature functioning mRNA molecule. Translation of the chemically stable mRNA molecule into a polypeptide occurs only after the molecule is transported into the cytoplasm. Functional proteins are usually produced after extensive post-translational modifications.

In general, four broad levels of gene regulation include transcriptional, post-transcriptional, translational, and post-translational. Transcriptional regulation can be attributed to any variable that influences the efficiency and/or rate of transcription or prevents transcription from occuring (e.g., an aberration within the coding portion of a locus, or the flanking sequences that influence its expression). Post-transcriptional regulation is any event that influences the splicing of pre-mRNA, pre-mRNA stability in the nucleus, nucleocytoplasmic transport, or stability of the RNA molecules in the cytoplasm. Translational regulation involves any variable that influences the translation efficiency of

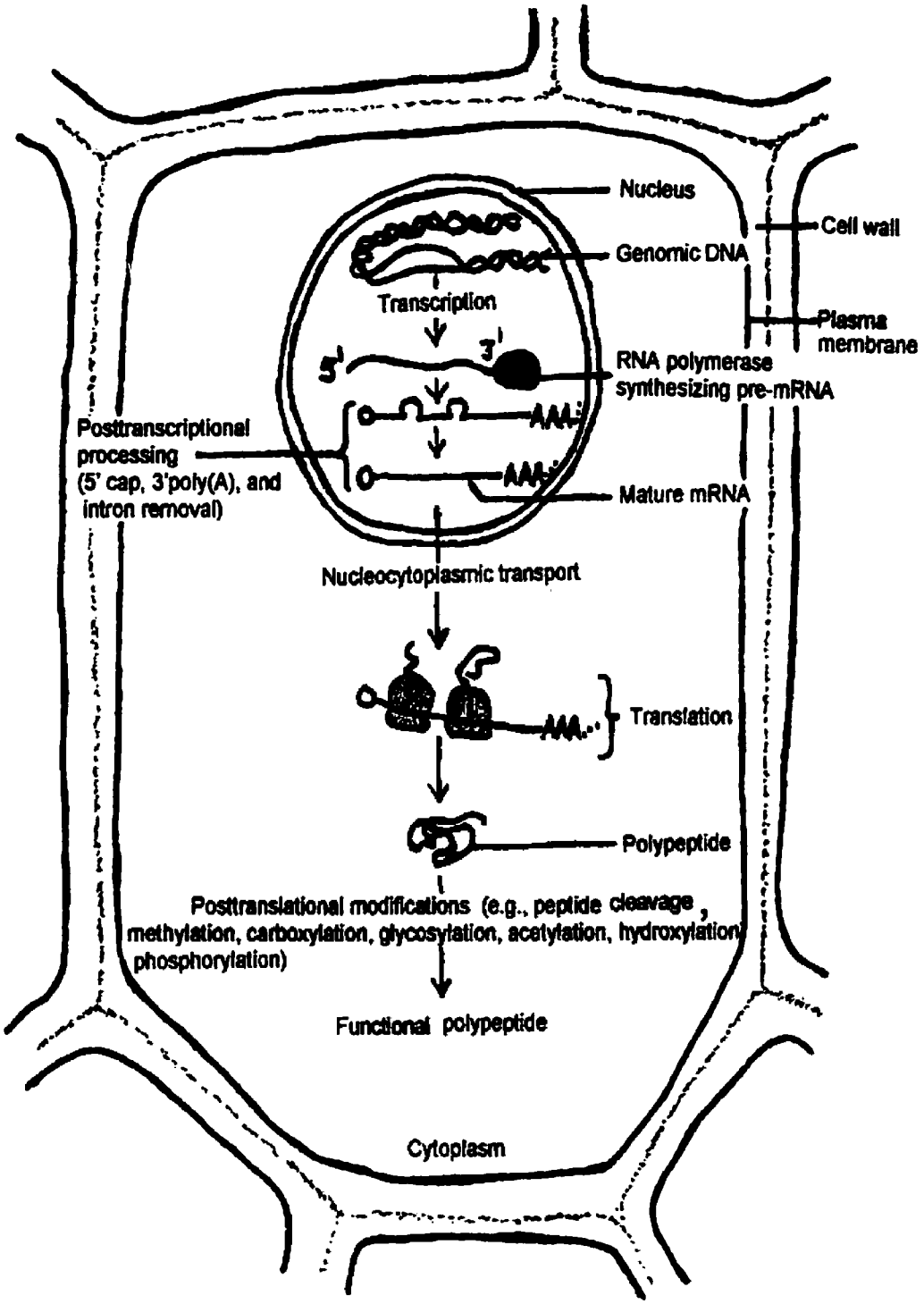


Fig. 2.7 Molecular processes involved in synthesizing a functional protein in a plant cell. Adapted from Farrell (1993).

the mature mRNA into a peptide or prevents translation from occuring (e.g., absence of regulatory factors and sequences acting in trans and cis or inaccessibility of mRNA to the protein translation machinery). Post-translational regulation is any event that influences the functionality of a protein (e.g., peptide cleavage, methylation, carboxylation, glycosylation, acetylation, hydroxylation, and phosphorylation). The biochemical processes directly and indirectly involved in producing biologically functional proteins in eukaryotes are far from clear and their complexity offers an infinite number of steps at which gene regulation can occur (Farrell, 1993).

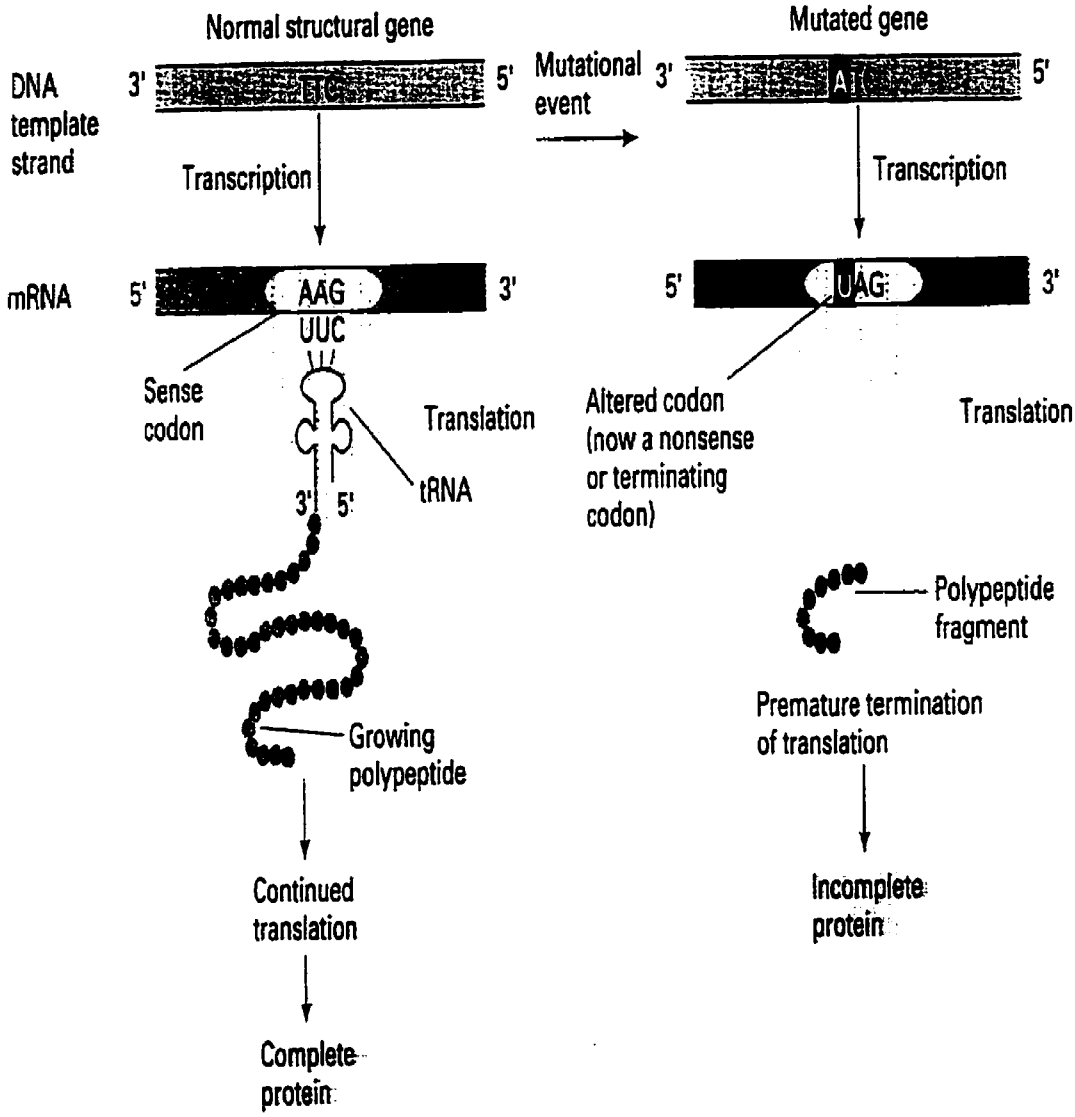
2.8 Gene Mutations

The existence of mutants indicates that alterations do occur in genes that often have such significant consequences to the organism that normal function is no longer possible (Russell, 1992). A mutation is any detectable and heritable change in the genetic material not caused by genetic recombination. Mutations can arise through changes at the base-pair level or at the chromosomal level (i.e., variation from the wild-type condition in either chromosome structure or number). A gene mutation occurs at the level of a gene and involves any one of a number of alterations of the DNA sequence of the gene, including base-pair substitutions (e.g., nonsense mutation) and additions or deletions of one or more base pairs (e.g., frameshift mutation). Gene mutations affecting a single base pair of DNA are called point mutations. A nonsense mutation is a base-pair change in the DNA that results in the change of an mRNA codon from one that specifies an amino acid to a chain-terminating (nonsense) codon (UAG, UAA, or UGA; Fig. 2.8). A frameshift mutation results from the insertion or deletion of a base pair in a gene. Such insertions or deletions can shift the mRNA's reading frame by one base so that either 1) incorrect amino acids are translated into the encoded polypeptide resulting in a nonfunctional polypeptide or 2) a nonsense mutation occurs resulting in a prematurely terminated polypeptide.

Few mutations in wheat have been characterized to date at the molecular level; however, molecular characterization of mutations resulting in human diseases have been extensively studied (Watson *et al.*, 1992). For example, β-thalassemias is a disease caused by abnormal synthesis of globin chains. Four globin chains, two α-chains and two β-chains,

Fig. 2.8 Nonsense mutation. A nonsense mutation is a base-pair change in the DNA that results in the change of an mRNA codon from one that specifies an amino acid to a chain-terminating (nonsense) codon (UAG, UAA, or UGA). For example, a mutation in the DNA template strand from 3'-TTC-5' to 3'-ATC-5' would change the mRNA codon from 5'-AAG-3' (lysine) to 5'-UAG-3', which is a nonsense codon. A nonsense mutation gives rise to chain termination at an incorrect place in the polypeptide and consequently results in the premature termination of the polypeptide. Instead of complete polypeptides, polypeptide fragments (usually non-functional) are released from the ribosomes. Adapted from Russell (1992).

Nonsense mutation.



make up the tetrameric protein known as hemoglobin. Over 50 mutations have been found in the β-globin gene among them β^+-thalassemias, characterized by the production of reduced β-globin levels and β^0-thalassemias, characterized by a complete absence of β-globin synthesis. Mutation types resulting in β^+-thalassemias include mutations in consensus sequences adjacent to conserved 5' (/GT) or 3' (AG/) dinucleotide intron splice sites, mutations within introns or exons producing cryptic 5' or 3' intron splice sites which compete with normal splice sites, promoter mutations (relative to the start site), RNA-cleavage or polyadenylation signal mutations (e.g., AATAAA→AACAAA), and cap site mutations. In contrast, mutation types resulting in β^0-thalassemias include nonsence mutations, frameshift mutations, and splice junction mutations in the conserved 5' GT/ or 3' AG/ dinucleotides of introns. A generalized overview of the mechanism of pre-mRNA splicing is presented in Fig. 2.9. Pre-mRNA splicing is carried out in two steps within the spliceosome, a large RNA-protein complex, which contains four small ribonuleoprotein particles (U1, U2, U4/U6, and U5) and numerous protein factors (Filipowicz *et al.*, 1995; Simpson and Filipowicz, 1996; Brown, 1996; Schuler, 1998). In the first step of splicing, cleavage occurs at the intron 5' border with the formation of a 2'-5' phosphodiester bond between the first nucleotide of the intron (+1G) and a branch point located 10-50 nucleotides upstream of the 3' splice site. In the second step, cleavage at the 3' splice site releases the intron lariat and the exons are ligated together. Splice junction mutations in the conserved 5' GT/ or 3' /AG dinucleotides of introns typically result in the abolition of their use and in the activation of adjacent splice sites.

Few waxy mutants, producing undetectable or reduced levels of protein, in maize (Echt and Schwartz, 1981; Shure *et al.*, 1983; Sano, 1985; Klosgen *et al.*, 1986), rice, potato (Hovenkamp-Hermelink *et al.*, 1987; Visser *et al.*, 1989), and barley have been studied at the molecular level. *Waxy* allelle mutations have been characterized as caused by large insertions/deletions attributed to transposable elements in maize *Wx* genes (Wessler and Varagona, 1985). Unspliced intron 1 (1 kb) was detected in the *Wx* transcripts of waxy rice cultivars, possessing no amylose, Wx protein, or normal 2.3 kb *Wx* transcript (Wang *et al.*, 1995; Cai *et al.* 1998). Bligh *et al.* (1998) later demonstrated that a GT to TT mutation at the 5' /GT dinucleotide of intron 1 reduced the efficiency of *Wx* pre-mRNA

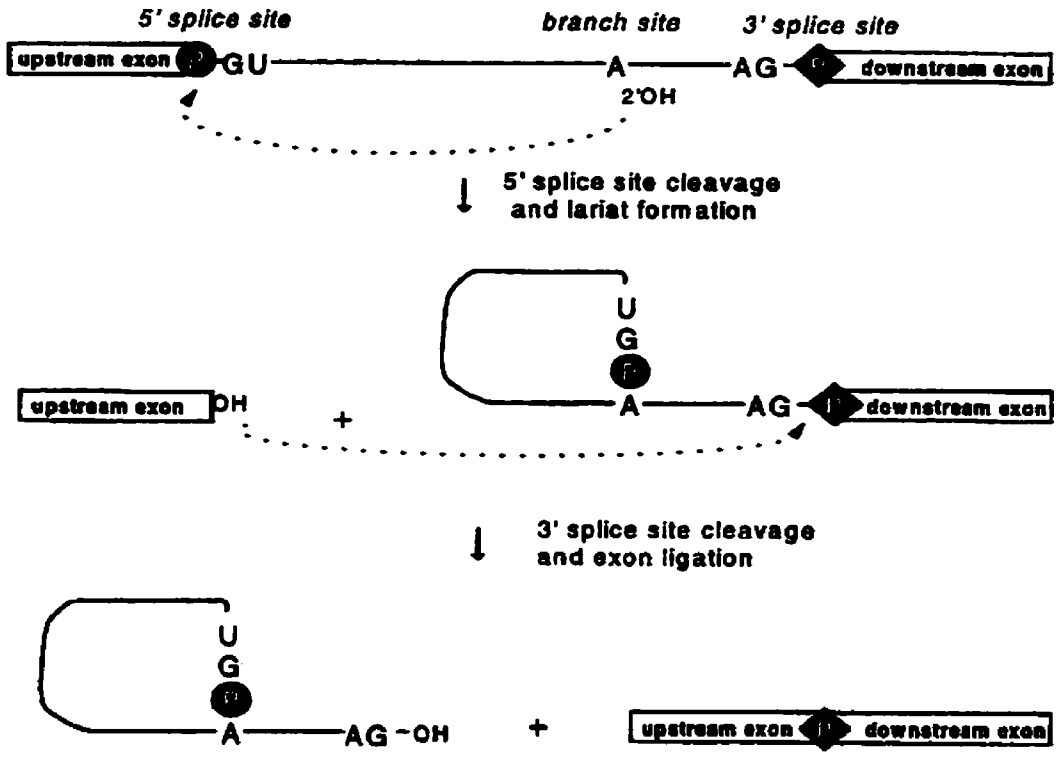


Fig. 2.9 The two transesterification steps used for spliceosome catalyzed pre-mRNA intron splicing. Conserved nucleotides and the phosphates at the splice sites are shown. The exons are shown as boxes and the introns as lines. The first transesterification reaction is attack by the 2'-hydroxyl (OH) group of an adenosine (A) ribose located 20-60 nt upstream from the 3' end of the intron, onto the 5' exon 1-intron boundary. This links the 5' end of the intron from the exon and links it to the A residue. The free OH group at the end of exon 1 generated by the initial reaction, carries out a second transesterification onto the 3' intron-exon 2 boundary. The final products of these two reactions are the joining of exons 1 and 2 and the liberation of the intron in the form of a lariat structure. The four small ribonuleoproteins (snRNPs) and the numerous protein factors required by these reactions are not shown. Adapted from Simpson and Filipowicz (1996).

processing and resulted in alternative splicing at three cryptic 5' splice sites. Isshiki *et al.* (1998) reported the inefficient excision of intron 1 from the 5' untranslated region of the *Wx* transcript of waxy rice and associated the GT to TT mutation with reduced *Wx* transcript and Wx protein levels. They studied a *Wx* allele (Wx^b) that resulted in a 10-fold decrease in *Wx* mRNA and protein production when compared to a wildtype *Wx* (Wx^a) allele. As previously reported by other groups, the Wx^b allele had the same GT to TT mutation at the 5' splice site of intron 1 and resulted in alternative splicing at three cryptic 5' splice sites. Isshiki *et al.* (1998) introduced single base mutations to the 5' splice sites of both the Wx^a (GT mutated to TT) and Wx^b (TT mutated to GT) alleles, fused them to the *GUS* reporter gene, and introduced them into rice protoplasts. Their results demonstrated that the low expression level of the Wx^b allele resulted from the single base mutation at the 5' splice site of intron 1. A frameshift mutation resulted in the pre-mature termination of translation of the Wx protein in waxy potato (van der Leij *et al.*, 1991b). A nonfunctional Wx protein with a molecular weight slightly higher than expected was detectable in the waxy potato tubers. These researchers hypothesized that the mutant (nonfunctional) Wx-protein was the product of the null *Wx* allele and its presence was explained if the re-initiation of translation took place at an internal, in phase, AUG codon. Domon (1996) has characterized barley lines possessing 2 to 10 % amylose and reduced Wx protein levels relative to normal barley lines. The study detected a 403 bp deletion in the mutant *Wx* allele, spanning from position -149 bp to +254 bp (+1 denotes transcription start site), that resulted in the loss of the original transcriptional start site. The presence of the nonfunctional Wx protein in the waxy barley line was explained if it was assumed that re-initiation of transcription took place at an internal transcriptional start site. The 403 bp deletion was suggested to reduce the rate of transcription of *Wx* mRNA transcripts leading to reduced Wx protein levels and amylose concentrations.

Recently, research has focused on the molecular characterization of the null *Wx-A1b*, *Wx-B1b*, and *Wx-D1b* alleles of a waxy hexaploid wheat line (Vrinten *et al.* 1999). The characterization of a 117 bp deletion at the 5' end of the *Wx-A1b* null allele in waxy wheat (Vrinten *et al.* 1999), derived by crossing Kanto 107 and Bai-Huo, is reviewed in chapter 4. Southern blot analysis suggested that the absent Wx-B1 protein in the waxy

line resulted from the deletion of the *Wx-B1* gene and/or the end of the long arm of chromosome 4A. A 588 bp deletion, including the 3' untranslated region and the polyadenylation signal, was detected at the 3' end of a *Wx-D1b* cDNA encoded by the null *Wx-D1b* allele of the waxy line. The aberrant *Wx-D1b* cDNA encoded a truncated Wx-D1 protein lacking the last 30 amino acids of the protein. The presence of an aberrant Wx-D1 protein with an altered molecular weight was not detected in the endosperm starch of the waxy line. Thus, the researchers concluded that the aberrant *Wx-D1b* cDNA in the waxy line was either not translated or the reduced abundance of *Wx* transcripts detected by Northern blotting lead to the translation of undetectable protein levels. Inefficient 3' end formation of aberrant *Wx-D1* transcripts was thought to contribute, at least in part, to the reduced *Wx* transcript levels in the waxy line.

3.0 ISOLATION AND CHARACTERIZATION OF A cDNA ENCODING A GRANULE-BOUND STARCH SYNTHASE I (WAXY) PROTEIN ORIGINATING FROM GENOME D OF HEXAPLOID WHEAT

3.1 Abstract

Isolation of cDNA encoding enzymes that synthesize the amylose or amylopectin component of starch are required for modifying starch structure using genetic engineering techniques. The objective of this study was to isolate and characterize a wheat cDNA encoding a Wx protein. A λ-ZAPII-cDNA library prepared from wheat kernels (*Triticum aestivum* L.; AABBDD) was screened using a digoxigenin labeled 911 bp probe. A 2.2 kb cDNA clone denoted GBSSIMMI was sequenced. The translated region of the cDNA predicted a 604 amino acid Wx peptide (66.3 kD) that did not possess an 11 amino acid wheat specific insertion (AMLCRAVPRRA). The deduced amino acid sequence showed 94 % similarity with a wheat Wx-A1 peptide, 96 % similarity with a wheat Wx-B1 peptide, and 100 % identity with two wheat Wx-D1 peptides. Over expression of the mature 60.1 kD Wx-D1 peptide, encoded by GBSSIMMI, in a glycogen synthase deficient *Escherichia coli* strain was detected with antiserum prepared using a wheat Wx peptide. Complementation analysis and an *in vitro* amylopectin-primed starch synthase assay were unable to detect a biologically active Wx protein using *E. coli* cells or soluble cell extracts containing the over expressed Wx-D1 protein, respectively. Deduced Wx peptide N-terminal sequences, isoelectric points, molecular weights, and amino acid sequence similarities indicate that clone GBSSIMMI encodes a Wx-D1 peptide originating from genome D of hexaploid wheat.

3.2 Introduction

Starch, a food reserve in plants, is stored in various starch storage organs such as the endosperm of cereals, roots of cassava, and tubers of potato (Preiss and Sivak, 1996). In cereals, starch consists of a mixture of ~ 25 % amylose and ~ 75 % amylopectin polymers

which plants synthesize and store within amyloplasts as insoluble granules. Glucose is the basic building block of both amylose and amylopectin. Alpha-amylose is a primarily linear polymer made up of several thousands of α-1,4-linked glucose residues. In contrast, amylopectin is a highly branched polymer consisting of α-1,4 linked glucose residues with frequently occuring α-1,6 branches of glucose residues. Many of the properties of starches that determine their suitability for particular end-uses are dependent upon their amylose/amylopectin ratios (Gibson *et al.*, 1997).

The final steps of starch biosynthesis involve four enzymes, including ADP-glucose pyrophosphorylase (ADP-GPPase; EC 2.7.7.23), starch synthases (granule-bound starch synthase and soluble starch synthase; EC 2.4.1.21), starch branching enzymes (EC 2.4.1.28) (Preiss and Sivak, 1996), and debranching enzymes (EC 3.2.1.41 and EC 3.2.1.68; Mouille *et al.*, 1996). ADP-Glucose, the basis of both amylose and amylopectin molecules, and a liberated pyrophosphate are formed from ADP and glucose-1-phosphate by ADP-GPPase. Starch synthases form α-1,4 linkages between the non-reducing end of a glucose polymer and ADP-glucose molecules. *In vitro*, it is has been shown that starch synthases use both amylose and amylopectin as substrates (Denyer *et al.*, 1996). Lastly, starch branching enzymes catalyze the formation of α-1,6 linkages between starch polymers, whereas debranching enzymes hydrolyze α-1,6 bonds. Of the multiple isoforms of starch synthases in plants, including soluble starch synthases (Li *et al.*, 1999) and granule-bound starch synthases, the GBSS I isoform commonly known as the Wx protein is primarily responsible for the synthesis of amylose polymers in starch. Since the Wx protein is absent in waxy mutants (≤ 1 % amylose and ≥ 99 % amylopectin) it has been assumed that the Wx protein is responsible for synthesizing amylose in plants (Yamamori and Nakamura, 1994). Evidence for this association has been offered by antisense inhibition of the *Wx* transcript in potato tubers resulting in the elimination of amylose and the Wx protein (Kuipers *et al.*, 1995) and expression of a *Wx* gene in an amylose-free potato mutant resulting in restoration of amylose synthesis (Flipse *et al.*, 1994).

In hexaploid wheat, three Wx proteins known as Wx-A1, Wx-B1, and Wx-D1 are encoded by loci on chromosomes 7AS, 4AL (translocated form 7BS), and 7DS, respectively (Chao *et al.*, 1989; Ainsworth *et al.*, 1993). Recently, genomic DNA sequences encoding Wx-A1, Wx-B1, and Wx-D1 proteins in hexaploid wheat have been

isolated and characterized (Murai *et al.*, 1999). Deduced molecular weights of 59.0 kD for a Wx-A1 protein (accession no. AB019622), 58.8 kD for a Wx-B1 protein (accession no. AB019623), and 58.9 kD for a Wx-D1 protein (accession no. AB019624) were predicted from the isolated *Wx* genes. In addition, these peptides have slightly different isoelectric points (pH at which protein molecules carry no net charge), ranging from ~ 6.5 to 5.5 (Wx-A1 has the most basic pI, Wx-D1 has an intermediate pI, and Wx-B1 the most acidic pI; Yamamori *et al.*, 1994). Northern blot analysis has established that the *Wx* genes of wheat produce 2.4 kb transcripts at high levels throughout grain filling, 5 to 25 days post anthesis (Ainsworth *et al.*, 1993; Vrinten *et al.*, 1999). A wheat cDNA clone encoding a Wx peptide has been isolated (Accession no. X57233, Clark *et al.*, 1991). The deduced peptide contains 615 amino acids (67.8 kD), including a transit peptide (7.7 kD) which targets the peptide to plastids (Klosgen *et al.*, 1989). Functional analysis of the transit peptide (van der Leij *et al.*, 1994) or the mature Wx-A1 peptide encoded by cDNA X57233 has not been performed and the functional significance, if any, of an 11 amino acid insertion (AMLCRAVPRRA) in the encoded peptide sequence remains unclear. Researchers have recently established that the *Wx* cDNA isolated by Clark *et al.* (1991) encodes a Wx-A1 peptide, a product of genome A of wheat (Vrinten *et al.*, 1999; Murai *et al.*, 1999). The objective of this study was to isolate and characterize a wheat cDNA encoding a Wx protein.

3.3 Materials and Methods

3.3 1 Plant Material

The wildtype alleles of the A, B, and D genomes are designated *Wx-A1a*, *Wx-B1a*, and *Wx-D1a*, respectively, whereas null alleles that produce no detectable Wx peptide are designated *Wx-A1b*, *Wx-B1b*, and *Wx-D1b*. Kernels of Bai-Huo, a Chinese line (*T. aestivum*; *Wx-A1a*, *Wx-B1a*, and *Wx-D1b* alleles), were surface sterilized using a 1 % sodium hypochlorite (bleach) solution and germinated in petri dishes at 4 °C for 30 days before transferring to soil. Plants were grown using the following greenhouse conditions: 28/18 °C (day/night), 16 h light (250 μmol m^{-2} s^{-1}) provided by banks of fluorescent tubes and incandescent bulbs, and 8 h dark. Plants were fertilized every two weeks using Plant Products 2.0-0.88-1.65 (N-P-K) at a rate of 3 g L^{-1} of water. Mature kernels were

harvested, stored at 4 °C until needed, and used for starch extraction as described by Zhao and Sharp (1996).

3.3.2 Screening of cDNA Library

A λ-ZAPII-cDNA library (Stratagene) prepared from poly A+ RNA of wheat kernels (cv Fielder; *Wx-A1a*, *Wx-B1b*, and *Wx-D1a* alleles, T. Demeke, personal communication) at 12 days post-anthesis (Nair *et al.*, 1997; Appendix 7.1) was screened. Approximately 1 x 10^5 pfu were screened as described by the ZAP-cDNA Synthesis Kit (Stratagene; Appendix 7.2). Plaque lifts were conducted using the protocol provided with Hybond-N+ membranes (Amersham). Digoxigenin plaque hybridization was conducted as described by Engler-Blum *et al.* (1993; Appendix 7.3) using a WxI probe (911 bp PCR product; see below) labeled with digoxigenin dUTP. Positive plaques were detected (Engler-Blum *et al.*, 1993), purified, and excised *in vivo* from the Uni-ZAP XR vector using the ExAssist/SOLR system (Stratagene). The longest cDNA was chosen for further characterization. The pBluescript SK(-) phagemid containing the 2.2 kb insert was designated GBSSIMMI. Plasmid DNA suitable for restriction analysis and DNA sequencing was isolated as described by del Sal *et al.* (1988; Appendix 7.4).

3.3.3 Digoxigenin (DIG) dUTP Labeled Probe

Phage containing cDNA library inserts packaged in Uni-ZAP XR vector were hydrolyzed in a 20 µL reaction volume containing: 0.5% (v/v) Tween 20, 1X PCR buffer (Boeh. Mann.), 1 µg/µL Proteinase K (10 mg/mL), and 10 µL phage lysate. Reactions were incubated at 65 °C (45 min), 95 °C (10 min), 4 °C (10 min) and centrifuged at 13000 x g at 4 °C (5 min). A 25 µL PCR reaction was prepared containing: 1X Taq DNA polymerase reaction buffer (Boeh. Mann.), 0.2 pmol/µL each of primer GBSSF2/GBSSR3 (Table 3.1; Appendix 7.5), 200 uM of each dNTP, 2 Units (U) Taq DNA Polymerase, and 5 µL of phage lysate (Appendix 7.6). Amplification conditions included 94 °C (5 min), followed by 29 cycles of 94 °C (1 min), 64 °C (1 min), and 72 °C (2 min). PCR amplification products (10 µL) were separated by 1.2 % agarose gel electrophoresis and visualized by ethidium bromide staining. The expected 911 bp PCR product was purified from the gel and ligated into pCR2.1 (Marchuk *et al.*, 1991), using

the Original TA Cloning Kit (Invitrogen; Appendix 7.7), to give pWxI. Digoxigenin (DIG) dUTP labeling of the WxI fragment was performed in a 50 µL final reaction volume containing: 1X Taq DNA polymerase reaction buffer (Boeh. Mann.), 0.2 pmol/µL each of primers GBSSF2/GBSSR3, 200 uM of each dNTP (PCR DIG Probe Synthesis Kit, Boeh. Mann.; Appendix 7.8); 2 U Taq DNA polymerase; and 35 pg of pWxI. Conditions included 94 °C (5 min), followed by 25 cycles of 67 °C (30 sec), 72 °C (2 min), and 94 °C (30 sec). The DIG labeled WxI probe (911bp) was stored at 4 °C, without further purification.

3.3.4 DNA Sequence Analysis

DNA sequencing reactions were performed using the Applied Biosystems Prism Dye-deoxy Cycle Sequencing Kit (Appendix 7.9). The reactions were run on an Applied Biosystems Prism 373 DNA sequencer (Perkin-Elmer). Sequence was initiated from known vector sequences. Based on these runs nested primers were constructed to extend the DNA sequence (Table 3.1). Both strands of the cDNA were completely sequenced. Primers were synthesized by the DNA Technologies Unit, National Research Council, Saskatoon, Canada using a Beckman Oligo 1000M DNA synthesizer. (Applied Biosystems; Appendix 7.10). DNA and deduced amino acid sequences were examined with the MEGALIGN program of DNASTAR (Lasergene Biocomputing software; Appendix 7.11) using a PAM 250 residue weight table and Cluster method (pairwise alignment parameters: K-tuple = 1 and gap penalty = 3; multiple alignment parameters: gap penalty =10 and gap length penalty = 10).

3.3.5 Construction of pM*Wxl* Expression Plasmid for Expression of the Waxy Protein in *Escherichia coli*

For expression of the Wx protein, without the 7.4 kD transit peptide, site directed mutagenesis was used to incorporate a unique Eco RI restriction site within WxIMM1 at bp 349 using the following mutant primers: WxMUTF1 (5'-GCGGTGCGAATTCATGGTGG-3') and WxMUTR1 (5'-CCACCATGAATTCGCACCGC-3') as described by the Quickchange site directed mutagenesis kit (Stratagene; Appendix 7.12). The insert coding

Table 3.1. DNA sequences of primers specific to waxy wheat cDNA clone denoted GBSSIMMI (Accession no. Y16340).

Name	Sequence	Direction	Position
MM2FP	5'-CGGCATGAACCTCGTGT-3'	Forward	380-397
MM3FP	5'-GGCAAGACCAAGGAGAA-3'	Forward	663-679
GBSSF2	5'-CTTCTGGCCTGCTACCT-3'	Forward	852-868
MM4FP	5'-GACTTCGCGCAGCTCAA-3'	Forward	963-979
MM5FP	5'-GGAAGGCGCTGAACAAG-3'	Forward	1276-1292
MM6FP	5'-TGGCTCACCAGATGATG-3'	Forward	1540-1556
MM7FP	5'-GGACGTGCTTCTGGAAC-3'	Forward	1874-1890
MM10FP	5'-CCTTGTACTGGTCGTAG-3'	Reverse	512-528
MM11FP	5'-ACACGAACACCACGTCC-3'	Reverse	851-831
MM12FP	5'-ATGAGCTCCTCCGCGTA-3'	Reverse	1110-1126
MM13FP	5'-ACGTCCTCCTCCTTCAG-3'	Reverse	1413-1429
MM14FP	5'-TGCAATCGACACTGAGC-3'	Reverse	1712-1728
GBSSR3	5'-ACCACCTTCTTCACGTCG-3'	Reverse	1745-1762
MMRTRP1	5'-CTACAACAAGCGGCTATCTC-3'	Reverse	2037-2056

for the mature Wx peptide (58.9 kD), excluding the transit peptide, was subcloned as a 1.9 kb Eco RI/Kpn I fragment from pBluescript (SK-) phagemid to the Eco R1/Kpn 1 sites of bacterial expression vector pKK388-1 (Clontech) to give pM*WxI*. The reconstructed gene coding for the Wx peptide was sequenced to verify that no unwanted mutations had occurred during this modification step. The modified N-terminus encoded by pM*WxI* adds 9 amino acids (MAAEFMVVRATGSGGMNLV; ~1.2 kD) to the unmodified mature N-terminus of the Wx peptide (ATGSGGMNLV).

3.3.6 Complementation Analysis of a Glycogen Synthase Deficient *E. coli* Strain

The pM*WxI* expression plasmid was used to complement a glycogen synthase deficient mutant of *E. coli*, strain RH98 (MC4100 *glg*A::Tn10), using standard techniques (Muffler *et al.*, 1997; Appendix 7.13). The wildtype *E. coli* strain JM101 was used as a positive control. Transformed cells were plated on nitrogen limiting glucose enriched medium (0.85 % KH_2PO_4, 1.1 % K_2HPO_4, 0.6 % yeast extract [Difco], 1.5 % agar [Difco], pH 7.0) containing 1 % glucose, 100 mg/L ampicillin, and 1 mM isopropyl-β-D-thiogalactopyranoside [IPTG] (Govons *et al.*, 1969). Cells were plated and incubated at 37 °C for 20 h. Differences in their ability to accumulate glycogen, a storage carbohydrate in bacteria, were detected by staining cell colonies with Lugol's iodine solution (0.03 M I_2, 0.04 M KI; 5 mL per plate). Colonies differing in their ability to accumulate glycogen after iodine staining were divided into three classes: colorless (did not accumulate glycogen), blue (accumulated a small amount), and dark brown (accumulated a large amount).

3.3.7 Expression of the Waxy Protein in *E. coli*

Bacterial cells were grown at 37 °C in 45 mL LB medium containing 100 mg/L ampicillin to an absorbance at 600 nm of 0.6 followed by induction of the *lac* promoter by addition of IPTG to a final concentration of 1 mM. Cell growth was continued for another 4 h at 27 °C, after which the cells were collected by centrifugation at 12 000 x g for 10 min at 4 °C, resuspended in 1 mL of ice-cold extraction buffer (50 mM Tris-acetate pH 8.5, 10 mM EDTA, 5 mM DTT, 100 µg/mL PMSF, and 2 µg/mL leupeptin), and lysed by sonication. Cell debris was removed from lysate by centrifugation at 15 000

x g for 15 min at 4 °C. The protein content of the soluble cell extract was determined using a dye-binding protein assay (Bradford Kit; Bio Rad; Appendix 7.14). Soluble cell extracts were used immediately in starch synthase assays.

3.3.8 SDS-PAGE and Immunoblotting

Purified starch granules (10 mg) were resuspended in 280 µL wash buffer II (0.625 M Tris-HCl, 2.3 % [w/v] SDS, 10 % [v/v] glycerol, and 0.005 % [w/v] bromophenol blue) and 20 µL β-mercaptoethanol followed by a 10 min boil, 5 min on ice, and 20 min centrifugation at 15 000 x g to pellet the gelatinized starch (Appendix 7.15). The supernatant was loaded onto 16 % SDS polyacrylamide gels (Zhao and Sharp, 1996; Appendix 7.16) to be immunoblotted (10 µL/lane) or Coomassie Brilliant Blue R stained (70 µL/lane; Sambrook *et al.*, 1982; Appendix 7.17). Soluble protein extract (60 µg/lane; see previous section) was resuspended in wash buffer II and 5 µL β-mercaptoethanol, boiled for 5 min, and loaded on to denaturing gels for electrophoresis.

Proteins were transferred to immobilon-P membrane (Millipore; Appendix 7.18) by electroblotting in transfer buffer (25 mM Tris base, 192 mM glycine, 0.05 % [w/v] SDS, 20% [v/v] methanol) at 35 V with 4 °C cooling for 5 h. Blots were incubated in blocking buffer (100 mM Tris HCl pH 7.5, 0.9 % NaCl [w/v], 0.1 % [w/v] Tween-20, 5 % [w/v] carnation milk) for 2 h. Blots were incubated with antiserum (1:2000 dilution in blocking buffer) for 3 h followed by 4 x 15 min washes in blocking buffer then incubated with alkaline phosphatase-conjugated antibody (1:5000 dilution in blocking buffer; Stratagene; Cat. no. 200374) for 3 h. Antiserum was prepared using the denatured 59-60 kD Wx-D1 peptide isolated from wheat endosperm starch (Demeke *et al.*, 1997a). Blots were washed 4 x 15 min in blocking buffer and 1 x 15 min in blocking buffer without Tween-20 before incubating in alkaline phosphatase developing solution (100 mM Tris HCl 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 0.4 % (v/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Stratagene; Cat. no. 300045) and 0.7 % (v/v) nitroblue tetrazolium (NBT; Stratagene; Cat. no. 300130).

3.3.9 *In Vitro* Starch Synthase Enzyme Assay

The starch synthase activity in *E. coli* cell extracts (Appendix 7.19) was determined using an amylopectin primed starch synthase assay (Denyer *et al.*, 1995; Appendix 7.20). The transfer of glucose onto amylopectin primer from ADP (^{14}C) glucose was measured in a total reaction volume of 200 µL containing: 3, 15, 30, or 60 µg of soluble cell extract, 0.1 M bicine pH 8.5, 25 mM potassium acetate, 10 mM glutathione, 5 mM EDTA, 10 mM DTT, 1 mg potato amylopectin (Sigma Type III), and 70 nmol adenosine diphospho-D-[U-^{14}C] glucose (specific activity = 627 cpm/nmol, Amersham). Assay mixtures containing heat-denatured soluble cell extracts were used as negative controls. The enzyme reaction was initiated by the addition of the ADP (^{14}C) glucose, incubated at 25 °C for 30 min, and terminated by heating at 100 °C for 2 min. Aliquots of 100 µL were removed from each reaction and absorbed on Whatman 31 ET filter discs, which were washed four times for 30 min with a 75 % (v/v) methanol and 1 % KCl (w/v) solution to remove unincorporated ADP (^{14}C) glucose. The filter discs were air-dried and the amount of glucan synthesized was determined using a scintillation counter. The starch synthase activity was calculated as nmol of glucose incorporated into glucan per milligram of protein per min after subtraction of background values obtained from heat denatured extracts. Average activity values are based on triplicate determinations. SAS programs and procedures (SAS Institute, Cary, NC) were used in the statistical analyses. Unpaired t-tests were used to compare means from enzyme determinations.

3.4 Results and Discussion

3.4.1 Isolation and Characterization of a Wheat cDNA Clone Encoding a Waxy Protein

The 2245 bp cDNA in clone GBSSIMMI includes an open reading frame of 1812 nucleotides from the initiating ATG at position 156 to the termination codon, TGA, at position 1968 (Fig. 3.1). The cDNA includes a 5' untranslated region of 155 bp, a 3' untranslated region of 275 bp, and putative poly adenylation signals at positions 2199 and 2206 preceding a poly (A) tail of 21 residues (Fig. 3.1). The deduced peptide (604 amino acids; 66.3 kD) includes a putative N–terminal transit peptide (70 amino acids; 7.4 kD)

thought to direct the peptide to the plastids (Klosgen *et al.*, 1989; de Boer *et al.*, 1991). The predicted cleavage site of the transit peptide is based on N-terminal sequencing of the three mature Wx peptides of hexaploid wheat (Nakamura *et al.*, 1995a).

Comparison of deduced Wx peptide N-terminal sequences, isoelectric points, molecular weights, and amino acid sequence similarities of the Wx-A1 peptide encoded by cDNA X57233 with the Wx peptide of cDNA Y16340 suggest that the latter sequence may encode a Wx-D1 peptide. The 14 N-terminal amino acids of the mature peptides Wx-A1 and Wx-D1 are identical (ATGSGGMNLV) (Nakamura *et al.*, 1995a; Murai *et al.*, 1999). Only the N-terminal amino acid sequence of Wx-B1 varies relative to the other two peptides by replacing a glycine residue with an alanine residue in the fifth postion (ATGSAGMNLV). The deduced mature Wx-D1 peptide sequence of cDNA Y16340 (534 amino acids; 58.9 kD) includes 61 strongly basic (positively charged; K, R); 70 strongly acidic (negatively charged; D,E); 194 hydrophobic (A, I, L, F, W, V); and 111 polar residues (N, C, Q, S, T, Y). In contrast, the mature Wx-A1 peptide encoded by cDNA X57233 (545 amino acids; 60.1 kD) includes 64 strongly basic; 70 strongly acidic; 200 hydrophobic; and 112 polar residues. The deduced isoelectric point (pI) of the Wx-A1 peptide encoded by cDNA X57233 (pI = 6.1) was more basic relative to the pI of the peptide encoded by GBSSIMMI (pI = 5.6) which is expected when comparing the isoelectric points of either Wx-D1 or Wx-B1 peptides with the Wx-A1 peptide (Yamamori *et al.*, 1994). The deduced mature Wx-A1 peptide encoded by cDNA X57233 (60.1 kD) has a slightly higher molecular weight relative to the mature Wx-D1 peptide encoded by cDNA Y16340 (58.9 kD). This difference agrees with the general trends detected in mobility when comparing the Wx-A1 and Wx-D1 peptides (Zhao and Sharp, 1996; Murai *et al.*, 1999). The mature Wx-D1 peptide encoded by cDNA Y16340 has the same molecular mass (58.9 kD) as the Wx-D1 peptide encoded by the *Wx-D1* gene (Accession no. AB019624). The Wx-D1 peptide encoded by cDNA Y16340 exhibits a high degree of similarity (> 80 %) with the amino acid sequences of *Wx* genes of monocots (Fig. 3.2). The deduced Wx-D1 peptide from cDNA Y16340 shows 94 % similarity with the Wx-A1 peptide encoded by cDNA X57233 (Fig. 3.2), 96 % similarity with the Wx-B1 peptide encoded by the *Wx-B1* gene (Accession no. AB019623; data not shown), and 100 %

Fig. 3.1 Nucleotide sequence of a wheat cDNA encoding a Wx-D1 peptide. The partial N-terminal sequence of the deduced peptide of the cDNA sequence is shown below the nucleotide sequence. The numbers on the left refer to the amino acid number and the numbers on the right refer to the number of nucleotides. The predicted transit peptide is underlined. The arrow denotes the predicted cleavage site of the transit peptide from the Wx peptide. The boxed nucleotides (20-mers) denote the region that was modified to incorporate a unique Eco R1 restriction site. Bolded nucleotides denote forward primer GBSSF2 (17-mers) and reverse primer GBSSR3 (18-mers). The translation initiation codon, termination codon, and putative poly adenylation signals are double underlined. The wheat cDNA sequence and deduced peptide sequences have been deposited in GenBank (Accession no. Y16340).

```
      AATTCGGCACGAGGAACAACAACAAGGACACTCACTCGCCAGTGCCCGGCCGGCGACTGT  60
      GAGTACGCACGCCGCCCGATCGTCCGTCCGTCCAAGAAGAAGAGGAGATCAGATCAGGCA 120
      TCTCTTGCTGCAGCTAGCCACACCCTGCGCGCGCCATGGCGGCTCTGGTCACGTCCCAGC 180
                                        M  A  A  L  V  T  S  Q
      TCGCCACCTCCGGCACCGTCCTCGGCATCACCGACAGGTTCCGGCGTGCAGGTTTCCATG 240
9     L  A  T  S  G  T  V  L  G  I  T  D  R  F  R  R  A  G  F  H
      GCGTGAGGCCCCGGAGCCCGGCGGATGCGGCTCTCGGCATGAGGACCGTCGGAGCTAGCG 300
29    G  V  R  P  R  S  P  A  D  A  A  L  G  M  R  T  V  G  A  S
      CCGCCCCAACGCAAAGCCGGAAAGCGCACCGCGGGACCCGGCGGTGCCTCTCCATGGTGG 360
49    A  A  P  T  Q  S  R  K  A  H  R  G  T  R  R  C  L  S  M  V
      TGCGCGCCACCGGCAGCGGCGGCATGAACCTCGTGTTCGTCGGCGCCGAGATGGCGCCCT 420
69    V  R↓A  T  G  S  G  G  M  N  L  V  F  V  G  A  E  M  A  P
      GGAGCAAGACCGGCGGCCTCGGCGACGTCCTCGGGGGCCTCCCCCCAGCCATGGCCGCCA 460
      ACGGCCACCGGGTCATGGTCATCTCCCCGCGCTACGACCAGTACAAGGACGCCTGGGACA 540
      CCAGCGTCGTCTCCGAGATCAAGGTCGTTGACAAGTACGAGAGGGTGAGGTACTTCCACT 600
      GCTACAAGCGCGGGGTGGACCGCGTGTTCGTCGACCACCCGTGCTTCCTGGAGAAGGTCC 660
      GGGGCAAGACCAAGGAGAAGATCTACGGGCCCGACGCCGGCACGGACTACGAGGACAACC 720
      AGCAGCGCTTCAGCCTTCTCTGCCAGGCGGCGCTGGAAGTGCCGAGGATCCTGAACCTCG 780
      ACAATAACCCCTACTTTTCTGGGCCCTACGGGGAGGACGTGGTGTTCGTGTGCAATGACT 840
      GGCACACGGGCCTTCTGGCCTGCTACCTCAAGAGCAACTACCAGTCCAATGGCATCTACA 900
      GGGCCGCAAAGGTGGCATTCTGCATCCACAACATCTCGTACCAGGGCCGCTTCTCCTTCG 960
      ACGACTTCGCGCAGCTCAACCTGCCCGACAGGTTCAAGTCGTCCTTCGACTTCATCGACG 1020
      GCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATGAAGGCCGGGATCCTGCAGG 1080
      CCGACAAGGTGCTGACGGTGAGCCCCTACTACGCGGAGGAGCTCATCTCTGGCGAAGCCA 1140
      GGGGCTGCGAGCTCGACAACATCATGCGCCTCACTGGGATCACCGGCATCGTCAACGGCA 1200
      TGGATGTTAGCGAGTGGGACCCCACCAAGGACAAGTTCCTCGCCGTCAACTACGACATCA 1260
      CCACCGCGTTGGAGGGGAAGGCGCTGAACAAGGAGGCGCTGCAGGCCGAGGTGGGGCTGC 1320
      CGGTGGACCGGAAGGTGCCCCTGGTGGCGTTCATCGGCAGGCTGGAGGAGCAGAAGGGCC 1380
      CCGACGTGATGATCGCCGCCATCCCGGAGATCCTGAAGGAGGAGGACGTCCAGATCGTTC 1440
      TCCTGGGCACCGGGAAGAAGAAGTTCGAGCGGCTACTCAAGAGCATTGAGGAGAAATTCC 1500
      CGAGCAAGGTGAGGGCCGTGGTCAGGTTCAACGCGCCGCTGGCTCACCAGATGATGGCCG 1560
      GCGCCGACGTGCTCGCCGTCACCAGCCGCTTCGAGCCCTGCGGCCTCATCCAGCTCCAGG 1620
      GGATGCGCTACGGAACGCCGTGCGCGTGCGCGTCCACCGGCGGGCTTGTCGACACGATCG 1680
      TGGAGGGCAAGACCGGGTTCCACATGGGCCGGCTCAGTGTCGATTGCAACGTGGTGGAGC 1740
      CGGCCGACGTGAAGAAGGTGGTGACCACCCTGAAGCGCGCCGTCAAGGTCGTCGGCACGC 1800
      CGGCATACCATGAGATGGTCAAGAACTGCATGATACAGGATCTCTCCTGGAAGGGGCCAG 1860
      CCAAGAACTGGGAGGACGTGCTTCTGGAACTGGGTGTCGAGGGGAGCGAGCCGGGGGTCA 1920
      TCGGCGAGGAGATTGCGCCGCTCGCCATGGAGAACGTCGCCGCTCCCTGAAGAGAGAAAG 1980
      AAAAGGAAGTTCTGGTGCATGGAGCGTCCATCCAGTCTGCAGGGTTCTCGTATGGGGAGA 2040
      TAGCCGCTTGTTGTAGCGAAGAAGGGCCGATATATATAATATATAGACTTATAAGTACTT 2100
      AACTTTTGTTGTGCCGCTTGCCTCTTTTACAAACAAAAAAGAAGTTAGGGGTTGTGCTTG 2160
      TTATAGTGTGCTGAACTGTGCTTGCATTTTGGTGTGGTATATTGCAATAAACAAAGGATT 2220
      TGTTAAAAAAAAAAAAAAAAAAAA                                     2245
```

	Wheat (X57233)	Barley (X07932)	Sorghum (U23945)	Maize (X03935)	Rice (X53694)	Snap dragon (AJ006293)	Sweet potato (U44126)	Potato (X58453)	Cassava (X74160)	Bean (AB029546)	Pea (X88789)	*E. coli* (J02616)
Wheat (Y16340)	94	95	82	82	81	64	63	62	61	59	59	28
Wheat (X57233)	-	93	80	79	79	61	60	60	60	57	56	27
Barley (X07932)		-	83	82	83	65	63	62	62	60	60	28
Sorghum (U23945)			-	94	84	65	65	62	63	60	60	28
Maize (X03935)				-	83	65	64	62	63	60	60	28
Rice (X53694)					-	63	63	62	62	61	61	27
Snap dragon (AJ006293)						-	79	79	78	70	69	25
Sweet potato (U44126)							-	79	74	68	69	26
Potato (X58453)								-	76	68	69	26
Cassava (X74160)									-	68	68	26
Bean (AB029546)										-	73	26
Pea (X88789)											-	26

Fig. 3.2 Sequence similarity (%) comparison among deduced amino acid sequences of waxy peptides. The putative transit peptide sequences are included in the comparison. GenBank accession nos. appear in parentheses.

identities with Wx-D1 peptides encoded by a null *Wx-D1b* allele (accession no. AF113844) and *Wx-D1* gene (accession no. AB019624). The Wx-D1 peptide encoded by cDNA Y16340 exhibits a low degree of similarity with the amino acid sequences encoded by *Wx* genes of dicots (< 60 %) and *E. coli* (28 %; Fig. 3.2). The trends in the comparisons with the Wx-A1 peptide and the 100 % identity among the Wx-D1 peptides offer evidence to support that cDNA Y16340 encodes a Wx-D1 peptide, a product of the D genome of hexaploid wheat.

Examination of the amino acid multiple alignment of two wheat cDNA and a barley cDNA illustrates that the protein encoded by cDNA X57233 contains an 11 amino acid insertion (AMLCRAVPRRA) between positions 219 and 230 (Fig. 3.3). Analysis of full-length Wx peptide sequences deduced from plant *Wx* genes to date (Fig. 3.2) confirms that the small insertion is unique to wheat (Ainsworth *et al.*, 1993). The mature Wx-A1 amino acid sequence encoded by cDNA X57233 (60.1 kD) is 100 % identical to the Wx-A1 peptide encoded by the *Wx-A1* gene characterized by Murai *et al.* (1999), except for the 11 amino acid insertion first described by Clark *et al.*(1991). Absence of the polymorphic insertion in the Wx-D1 protein encoded by cDNA Y16340 and the Wx-B1 protein encoded by the *Wx-B1* gene suggests that the small insertion may be uniquely encoded by alleles of the *Wx-A1* gene of hexaploid wheat (Matus *et al.* This study). The functional significance of this small insertion remains unclear. The three conserved regions among Wx peptides in plants (Fig. 3.3), including the KTGGL motif, thought to be the binding site for substrate ADP-glucose (Furukawa *et al.*, 1990), are maintained in the Wx peptides encoded by both wheat cDNA suggesting that the cDNA should encode functional Wx peptides.

3.4.2 Complementation of a Glycogen Synthase Deficient *E. coli* Strain and *In Vitro* Starch Synthase Enzyme Assay

Mutant RH98 and wildtype *E. coli* strains transformed with either pKK388-1 or pM*WxI* were stained with Lugol's iodine solution to visualize glycogen production (Fig. 3.4). The wildtype JM101 cells transformed with pKK388-1 stained reddish-brown with iodine detecting the production of glycogen. Mutant RH98 cells, transformed with

Fig. 3.3 Amino acid sequences of two independently isolated wheat cDNA (Accessions X57233 and Y16340) and a barley cDNA (Accession no. X07932). The predicted transit peptide sequence is underlined and its cleavage site is indicated by an arrow. The Wx-A1 peptide (67.8 kD) encoded by cDNA X57233 is presented directly above the Wx-B1 peptide encoded by the *Wx-B1* gene (Accession no. AB019623) and the Wx-D1 peptide (66.3 kD) encoded by cDNA Y16340. Amino acid numbering is as it appears in GenBank. Residues identical to the first sequence are indicated by an asterisk (*). The dashed (-) gap denotes amino acids absent in one of the sequences, but present in the others. Regions conserved among plant Wx peptides are bolded. The KTGGL motif is double underlined.

```
Wx-A1    1   MAALVTSQLATSGTVLSVTDRFRRPGFQGLRPRNPADAALGMRTVGASAAPKQ-SRKPH
Wx-B1    1   ****************GI******A****V***S****P*****T********Q***A*
Wx-D1    1   ****************GI******A**H*V***S*****************T*-***A*
X07932   1   ****A***********G*************************I********-***A*

Wx-A1    59  RFDRRCLSMVVR↓ATGSGGMNLVFVGAEMAPWSKTGGLGDVLGGLPAAMAANGHRVMV
Wx-B1    60  *GT********* ****A*************************P**********
Wx-D1    59  *GT********* *********************************P**********
X07932   59  *GS*****V**S ****-*****************************P**********

Wx-A1    116 ISPRYDQYKDAWDTSVISEIKVVDRYERVRYFHCYKRGVDRVFVDHPCFLEKVRGKTK
Wx-B1    117 ****************V*****A*E*********************************
Wx-D1    116 ****************V*******K*********************************
X07932   115 V*******************A*E*****F***********I***W**********

Wx-A1    174 EKIYGPDAGTDYEDNQQRFSLLCQAALEVPRILDLNNNPHFSGPYAMLCRAVPRRAGE
Wx-B1    175 ****************L**********A**********Y*****----------**
Wx-D1    174 *********************************N*D***Y*****----------**
X07932   173 ***************************A****N*****Y*****----------**

Wx-A1    232 DVVFVCNDWHTGLLACYLKSNYQSNGIYRTAKVAFCIHNISYQGRFSFDDFAQLNLPD
Wx-B1    222 ************************S*********************************
Wx-D1    221 ****************************A*****************************
X07932   220 ************************************************************

Wx-A1    290 RFKSSFDFIDGYDKPVEGRKINWMKAGILQADKVLTVSPYYAEELISGEARGCELDNI
Wx-B1    280 ************************************************************
Wx-D1    279 ************************************************************
X07932   278 ************************************************************

Wx-A1    348 MRLTGITGIVNGMDVSEWDPIKDKFLTVNYDVTTALEGKALNKEALQAEVGLPVDRKV
Wx-B1    338 ******************A*****AA*****************************
Wx-D1    337 ******************T*****A****I*************************
X07932   336 ******************T*****A****I*****A*******************

Wx-A1    406 PLVAFIGRLEEQKGPDVMIAAIPEIVKEEDVQIVLLGTGKKKFERLLKSVEEKFPTKV
Wx-B1    396 ************************L*****************************S**
Wx-D1    395 ************************L*********************I*****S**
X07932   394 ************************L*******I**********K****M*****G**

Wx-A1    464 RAVVRFNAPLAHQMMAGADVLAVTSRFEPCGLIQLQGMRYGTPCACASTGGLVDTIVE
Wx-B1    454 ************************************************************M*
Wx-D1    453 ************************************************************
X07932   452 ******************L*************************V**************

Wx-A1    522 GKTGFHMGRLSVDCNVVEPADVKKVVTTLKRAVKVVGTPAYHEMVKNCMIQDLSWKGP
Wx-B1    512 ************************************************************
Wx-D1    511 ************************************************************
X07932   510 ************************A***************Q*****************

Wx-A1    580 AKNWEDVLLELGVEGSEPGIVGEEIAPLALENVAAP.
Wx-B1    570 ******************VI********M*******.
Wx-D1    569 ******************VI********M*******.
X07932   568 ****************************M*******.
```

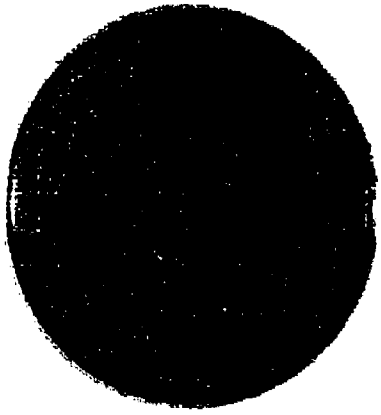


Fig. 3.4 Complementation analysis of starch synthase activity in *E. coli*. Photograph shows color development upon iodine staining (Govons *et al.*, 1969) of the following *E. coli* strains: A, glycogen synthase producing strain JM101 carrying pKK388-1; B, glycogen synthase deficient strain RH98 carrying pKK388-1; and C, RH98 carrying pM*Wx1*.

pKK388-1 vector, remained colorless when stained with iodine due to the absence of glycogen. Expression of pM*Wx1* in the mutant RH98 cells did not change the iodine staining phenotype from colorless to reddish-brown. Complementation of strain RH98 with pM*Wx1* was unsuccessful in indicating that the isolated cDNA was able to code for a functional Wx protein. The lack of (glycogen) primer in strain RH98 may explain why the Wx-D1 protein failed to reverse the mutant phenotype.

Recent reports have suggested that the Wx protein requires the crystalline matrix of amylopectin for binding *in vivo* (Dauvillée *et al.*, 1999). An *in vitro* amylopectin-primed starch synthase assay was used in an attempt to show that the isolated Wx wheat cDNA clone encoded an active Wx protein. Induction of expression in RH98 cells containing pM*Wx1* led to production of an ~ 60.1 kD Wx-D1 peptide which was recognized by antiserum made from the Wx-D1 peptide of wheat starch (Fig. 3.5). The expected molecular weight of the over expressed Wx-D1 peptide was 58.9 kD; however, the modified N-terminus of the deduced mature peptide encoded by pM*Wx1* (see methods section) added an additional 1.2 kD to the overall weight of the Wx-D1 peptide (60.1 kD). This slight increase in molecular weight makes it difficult to compare the relative mobility of the over expressed Wx-D1 protein in RH98 cells (Lane 2; Fig. 3.5B) with the mobility of Wx peptides from hexaploid wheat (Lane 1; Fig. 3.5B). RH98 cells transformed with pKK388-1 or pM*Wx1* lacked detectable starch synthase activity using 3, 15, 30, and 60 µg of soluble cell extract, respectively (Table 3.2). In JM101 cells transformed with pKK388-1, the positive control, high starch synthase activities were detected using all four protein extract levels. The high specific activities detected for the positive control reflect typical results seen in standard starch synthase assays using either purified (Imparl-Radosevich *et al.*, 1998) or cloned (Kumar *et al.*, 1986; Edwards *et al.*, 1996; Harn *et al.*, 1998; Knight *et al.*, 1998) soluble starch synthases. In contrast, the low specific activities generally detected in standard starch synthase assays using either purified (Smith, 1990; Denyer *et al.*, 1995) or cloned (Edwards *et al.*, 1995) Wx proteins have led some researchers to speculate that the Wx protein may require specific conditions for activity which are not available in standard amylopectin-primed starch synthase assays (Dry *et al.*, 1992). Thus, the biological activity of the Wx-D1 protein encoded by GBSSIMMI will have to be

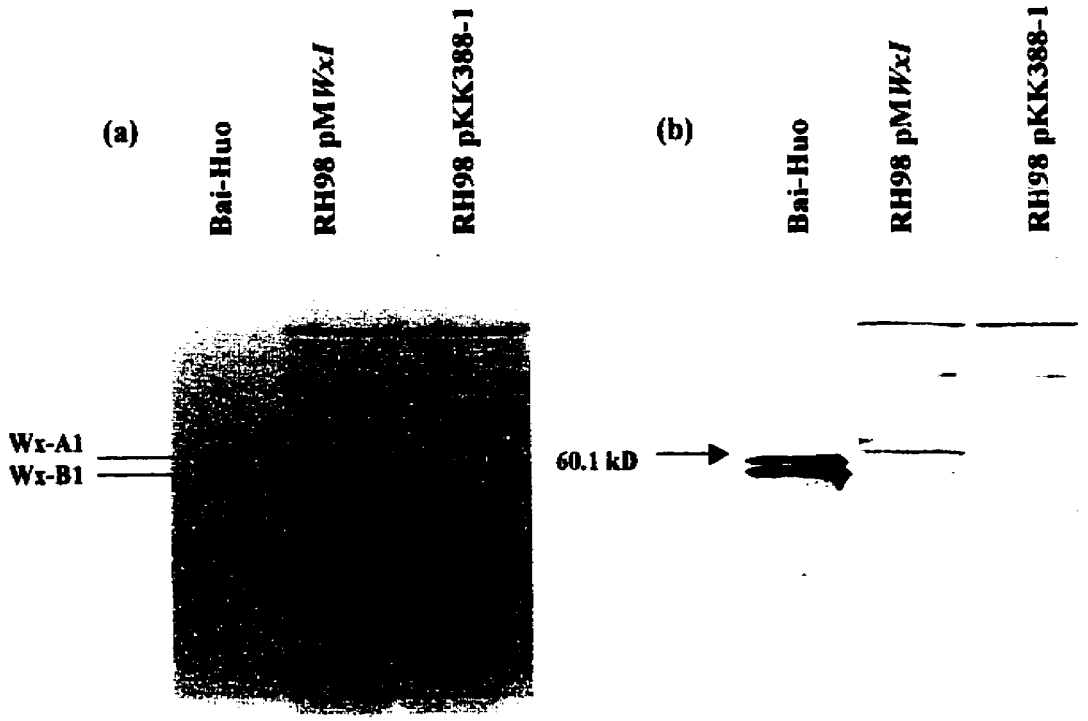


Fig. 3.5 Expression of the mature Wx-D1 wheat peptide in *E. coli*. (a) SDS-polyacrylamide gel. (b) Immunoblot. Lane 1, Wx-A1 and Wx-B1 peptides (~ 59-60 kD) from Bai-Huo wheat kernel starch; lane 2, RH98 *E. coli* cells transformed with pM*Wxl* expressing the Wx-D1 peptide (~ 60.1 kD); lane 3, RH98 *E.coli* cells transformed with pKK388-1 vector alone not expressing the Wx-D1 peptide. Note presence of a band ~ 60.1 kD in (2) of immunoblot but not (3) (arrow).

Table 3.2 Starch synthase activity (nmol [^{14}C] glucose incorporated mg protein^{-1} min^{-1} †) in *E. coli* strains.

	Soluble cell extract (µg/200 µL reaction)			
***E. coli* strain ‡**	**3**	**15**	**30**	**60**
RH98 pKK388-1	0 a	0 a	0 a	0 a
RH98 pM*Wx1*	0 a	0 a	0 a	0 a
JM101 pKK388-1	472 (± 16) b	166 (± 4) b	88 (± 0) b	45 (± 0) b

† Values are based on triplicate determinations.

‡ RH98 pKK388-1, Glycogen synthase deficient strain RH98 carrying pKK388-1; RH98 pM*Wx1*, strain RH98 carrying pM*Wx1*; JM101 pKK388-1, glycogen synthase producing strain JM101 carrying pKK388-1.

Numbers in parentheses are standard errors.

a-b Means within columns followed by the same letter are not significantly different (p=0.05), based on unpaired t-tests.

assessed *in vivo* using a Wx wheat line (possessing no Wx peptides and a reduced amylose concentration).

3.5 Conclusions

- For GBSSMMI, a 2.2 kb cDNA insert within pBluescript (Accession no. Y16340), the results reported herein indicate that the wheat clone encodes a 66.3 kD Wx peptide.

- The Wx peptide encoded by cDNA Y16340 showed 100 % sequence similarities with deduced null (Accession no. AF113844) and wildtype Wx-D1 peptides (Accession no. AB019624).

- Deduced Wx peptide N-terminal sequences, isoelectric points, molecular weights, and amino acid sequence similarities indicate that clone GBSSIMMI encodes a Wx-D1 peptide originating from the D genome of hexaploid wheat.

- Functional analysis assays in glycogen synthase deficient RH98 cells were unsuccessful in demonstrating that the over expressed Wx-D1 protein possessed biological activity.

- The biological activity of the Wx-D1 peptide encoded by GBSSIMMI should be assessed *in vivo* using a waxy wheat line.

4.0 ABERRANT *Wx* TRANSCRIPTS ENCODED BY THE NULL *Wx-A1b* ALLELE OF HEXAPLOID WHEAT

4.1 Abstract

Recent research has focused on the molecular characterization of null waxy (Wx) alleles, *Wx-A1b*, *Wx-B1b*, and *Wx-D1b*, that produce no detectable Wx proteins in the endosperm starch of allohexaploid wheat (*Triticum aestivum* L.; 2n=6x=42; AABBDD). The objective of this study was to identify aberrant *Wx* transcripts encoded by the null *Wx-A1b* allele of CDC Wx2, a waxy hexaploid wheat line, which result in an absent Wx-A1 protein (~59 kD). Two sister lines, CDC Wx2 and CDC Wx6 were obtained by crossing lines Bai-Huo (carries null *Wx-D1b* allele; lacks Wx-D1 protein) and Kanto 107 (carries null *Wx-A1b* and *-B1b* alleles; lacks Wx-A1 and -B1 proteins). Waxy protein profiling, amylose concentration determinations, Northern blot analysis, and reverse transcriptase PCR (RT-PCR) analysis were conducted. Ten RT-PCR derived cDNA clones were selected from each genotype and characterized by DNA sequencing analyses. The waxy phenotype of CDC Wx2, lacking Wx-A1, -B1, and -D1 proteins and possessing a reduced amylose concentration (~ 4 %), was associated with dramatically reduced levels of a 2.4 kb *Wx* transcript when compared to the higher levels in a wildtype control line. DNA sequencing of clones from Kanto 107 and CDC Wx2 characterized two types of aberrant *Wx* transcripts, one containing intron 1 and another containing introns 1 and 4. Intron 1 in both types of aberrant *Wx* transcripts contained a premature stop codon which resulted in the translation of a truncated Wx protein (~4 or 11 kD). Analysis of CDC Wx6, lacking Wx-B1 and -D1 proteins and possessing a reduced amylose concentration (~ 14 %), failed to reveal aberrant *Wx* transcripts, suggesting that the RNA defects in this study were not responsible for the absence of the Wx-B1 or -D1 proteins. Thus, the aberrant *Wx* transcripts were encoded by the null *Wx-A1b* allele. The presence of a premature stop codon in the *Wx* transcripts encoded by the null *Wx-A1b*

allele explains the absence of the ~59 kD Wx-A1 protein in CDC Wx2 and its parental line Kanto 107.

4.2 Introduction

In cereals, starch is a carbohydrate stored in water-insoluble granules as ~ 25 % amylose, α(1→4)-linked glucose chains, and ~ 75 % amylopectin, α(1→4)-linked glucose chains frequently branched by α(1→6)-linkages (Preiss and Sivak, 1996). Plants synthesize starch through a sequence of reactions involving four classes of enzymes: ADP-glucose pyrophosphorylase (EC 2.7.7.23), starch synthase (EC 2.4.1.21), starch-branching enzyme (EC 2.4.1.28), and starch-debranching enzyme (EC 3.2.1.41). Starch synthases catalyze the formation of the α(1→4)-linkages during the synthesis of amylose polymers. Of the multiple isoforms of starch synthases in plants, the granule bound starch synthase I isoform (GBSSI) also known as the Wx protein is primarily responsible for amylose synthesis (Kuipers *et al.*, 1995; Nelson and Rhines, 1962). In hexaploid wheat, the Wx-A1, Wx-B1, and Wx-D1 proteins encoded by homoeologous *Wx* genes located on the group 7 chromosomes (Zhao and Sharp, 1996; Chao *et al.*, 1989) are responsible for amylose synthesis in endosperm starch (Miura and Sugawara, 1996). The genomic DNA sequences of a *Wx-A1* gene encoding a 59 kD Wx-A1 protein (GenBank accession no. AB019622), a *Wx-B1* gene encoding a 58.8 kD Wx-B1 protein (accession no. AB019623), and a *Wx-D1* gene encoding a 58.9 kD Wx-D1 protein (accession no. AB019624) in hexaploid wheat have recently been isolated and characterized (Murai *et al.*, 1999).

The development of locally adapted wheat cultivars with altered amylose/amylopectin ratios involves the screening of germplasm to identify lines lacking Wx proteins (Graybosch, 1998). The wildtype alleles of the A, B and D genomes of wheat are designated *Wx-A1a*, *Wx-B1a*, and *Wx-D1a*, respectively, whereas null alleles that produce no detectable Wx protein are designated *Wx-A1b*, *Wx-B1b*, and *Wx-D1b* (Yamamori *et al.*, 1994). Wheat lines such as Bai-Huo (*Wx-A1a, Wx-B1a, Wx-D1b* alleles), lacking Wx-D1 protein, and Kanto 107 (*Wx-A1b, Wx-B1b, Wx-D1a* alleles), lacking Wx-A1 and Wx-B1 proteins, can be crossed to produce waxy lines (*Wx-A1b, Wx-B1b, Wx-D1b* null alleles), lacking three Wx proteins (Yamamori and Nakamura, 1994).

Waxy wheat carrying null alleles at all three *Wx* loci possess no Wx proteins and do not produce amylose in their endosperm starch (Yamamori *et al.*, 1995). Waxy and partially waxy lines with altered amylose/amylopectin ratios possess unique functional starch properties which may eventually make these starches suitable for novel niche markets (Graybosch, 1998).

The molecular characterization of the null *Wx-A1b* allele of a waxy wheat line derived by crossing Kanto 107 and Bai-Huo has recently been reported (Vrinten *et al.*, 1999). An aberrant *Wx-A1b* cDNA, encoded by the null *Wx-A1b* allele of the waxy line, contained a 117 bp deletion (accession no. AF113843). The 117 bp deletion resulted from the splicing of intron 1 through the use of a cryptic 5' GT splice site 117 bp upstream of the authentic 5' GT splice site identified in the wildtype *Wx-A1a* genomic sequence. The partial sequence of the null *Wx-A1b* genomic sequence of the waxy line contained a 23 bp exon-intron junction deletion, and subsequent 4 bp DNA filler insertion (nucleotides GGAC) at the 5' end of the deletion, which deleted the authentic 5' GT splice junction site of intron 1. The 117 bp deletion in the aberrant *Wx-A1b* cDNA resulted in a 39 amino acid deletion in the resultant Wx-A1 protein which included amino acids of the cleavage site of the transit peptide and the putative ADP-glucose binding site (KTGGL). The presence of a non-functional Wx-A1 protein with an altered molecular weight of ~63.3 kD was not detected in the soluble fraction of the endosperm or in the endosperm starch of the waxy line.

Intron splicing is carried out in two steps within the spliceosome, a large RNA-protein complex which contains four small ribonuleoprotein particles (U1, U2, U4/U6, and U5) and numerous protein factors (Schuler, 1998; Simpson and Filipowicz, 1996). The 5' splice dinucleotide /GT is conserved in 99 % of introns in monocots (/GC comprises the 5' splice site of the other 1% of introns). The 3' splice site dinucleotide, AG/, is invariant in higher plants. In the first step of splicing, cleavage occurs at the intron 5' border with the formation of a 2'-5' phosphodiester bond between the first nucleotide of the intron (+1 G) and a branch point located 10-50 nucleotides (nt) upstream of the 3' splice site. In the second step, cleavage at the 3' splice site releases the intron lariat and the exons are ligated together. Mutation of either the /GT or AG/ dinucleotide in plant introns typically result in the abolition of their use and the activation of cryptic splice sites.

The objective of our study was to identify aberrant *Wx* transcripts encoded by the null *Wx-A1b* allele of CDC Wx2, a waxy hexaploid wheat line derived by crossing Kanto 107 and Bai-Huo, which result in an absent Wx-A1 protein (~59 kD). Two previously unreported types of aberrant *Wx* transcripts encoded by the null *Wx-A1b* allele of waxy line CDC Wx2 have been identified. These *Wx* aberrant transcripts contain an in-frame premature stop codon (TAA) in the 5' region of the *Wx* transcripts which has been incorporated into the open reading frame of the protein by the presence of unspliced intron 1. The effects of *Wx* transcripts containing introns and premature stop codons on RNA transcript stability and Wx protein production are discussed.

4.3 Materials and Methods

4.3.1 Plant Material

Fully waxy wheat, denoted CDC Wx2 (*Wx-A1b*, *Wx-B1b*, and *Wx-D1b* null alleles), was produced by crossing Bai-Huo (*Wx-A1a*, *Wx-B1a*, and *Wx-D1b* alleles), a Chinese line, to Kanto 107 (*Wx-A1b*, *Wx-B1b*, and *Wx-D1a* alleles), a Japanese line (Demeke *et al.*, 1999). Two sister lines, CDC Wx2 and CDC Wx6 (*Wx-A1a*, *Wx-B1b*, and *Wx-D1b* alleles), both in the F_6 generation, were used in this study. CDC Wx6 was not initially included in the study, but was included only after the detection of aberrant mRNA transcripts in CDC Wx2. CDC Teal (*Wx-A1a*, *Wx-B1a*, and *Wx-D1a* alleles; T. Demeke, personal communication), a Canadian hard red spring wheat cultivar, was used as a positive control in Northern blot and RT-PCR analyses. The kernels of individual plants were harvested and threshed from each line. A seed source for each line was established from kernels of a single plant. All kernels with a vernalization requirement, except CDC Teal, were surface sterilized using a 1 % sodium hypochlorite (bleach) solution and germinated at 4 °C for 30 days in a petri dish before transferring to soil. Plants were grown using the following greenhouse conditions: 28/18 °C (day/night) and 16 h of light (250 μmol m^{-2} s^{-1}) provided by banks of fluorescent tubes and incandescent bulbs. Plants were fertilized every two weeks using Plant Products 2.0-0.88-1.65 (N-P-K) at a rate of 3 g L^{-1} of water. Kernels were collected at 5, 10, and 15 days post anthesis (DPA), frozen in liquid nitrogen, and stored at - 80 °C until needed.

4.3.2 Starch Extractions, SDS-PAGE, and Amylose Determinations

Starch granule extractions and 1D-SDS-PAGE was performed as described by Zhao and Sharp (1996). Purified starch granules (10 mg) were resuspended in 140 μL wash buffer II [0.6 mol L^{-1} Tris-HCl, 2.3 g L^{-1} SDS, 100 g L^{-1} glycerol, and 0.05 g L^{-1} bromophenol blue] and 10 μL β-mercaptoethanol. Starch samples were boiled (10 min), cooled on ice (5 min), and centrifuged (20 min) at 15 000 x g to pellet the gelatinized starch. To visualize the Wx proteins 17 μL of supernatant was loaded per lane onto gels to be silver stained (Bio-Rad, Mississauga, ON; Appendix 7.21). True amylose concentrations, obtained using defatted starch samples, were measured using purified starch granules based on the procedure described by Gibson *et al.* (1997; Appendix 7.22). SAS programs and procedures (SAS Institute, Cary, NC) were used in the statistical analyses. Unpaired t-tests were used to compare means from amylose determinations.

4.3.3 Northern Blot Analysis

Total RNA was isolated from immature endosperm tissue at 5, 10, and 15 DPA using TRIZOL Reagent (GibcoBRL Life Technologies, Burlington, ON) according to the manufacturer's instructions (Appendix 7.23). Total RNA (5 μg) was separated using 1.2 % agarose-formaldehyde gel electrophoresis and transferred on to Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ; Appendix 7.24). Digoxigenin (DIG)-dUTP hybridization and detection was performed as described by Engler-Blum *et al.* (1993) using a 911 bp fragment amplified from *Wx* wheat cDNA clone denoted GBSSIMMI (Accession No. Y16340; Matus Chapter 3). The 911 bp fragment was DIG-dUTP labeled using a PCR DIG Probe Synthesis Kit (Boehringer Mannheim, Laval, QB). Digoxigenin dUTP labeling of the 911 bp fragment was performed in a 50 μL final reaction volume containing: 1X Taq DNA polymerase reaction buffer (Boeh. Mann.), 0.2 pmol/μL each of primers GBSSF2/GBSSR3 (Table 3.1), 200 μM of each dNTP (PCR DIG Probe Synthesis Kit, Boeh. Mann.), 2 Units (U) Taq DNA polymerase, and 35 pg of GBSSIMMI. PCR amplification conditions included 94 °C (5 min), followed by 25 cycles of 67 °C (30 sec), 72 °C (2 min), and 94 °C (30 sec).

4.3.4 Reverse Transcriptase PCR Experiments

Primers were designed using Primer Designer version 2.0 (Scientific and Educational software) corresponding to regions on published *Wx* cDNA of hexaploid wheat (Accession nos. X57233, Clark *et al.*, 1991 and Y16340, Matus Chapter 3). Primers were synthesized using a Beckman Oligo 1000M DNA synthesizer. Based on the two *Wx* cDNA clones, PCR products ranging from 1383 or 1416 bp were expected using primer set MM2FP/GBSSR3 and 1200 bp using primer set GBSSF2/MMRTRP1. A generalized schematic diagram of the *Wx* genes of hexaploid wheat (Fig. 4.1) has been shown to illustrate that primer sets were designed to detect aberrations in regions encoding mature Wx proteins, produced after the cleavage of their transit peptides.

One μg of total RNA isolated from kernels at 10 DPA was used to synthesize first-strand cDNA using Oligo (dT) and Superscript II reverse transcriptase as described by the Superscript Pre-amplification System for First Strand cDNA Synthesis Kit (GibcoBRL; Appendix 7.25). The first PCR amplifications were performed on a PTC-100 MJ Research DNA Thermal Cycler with 4 μL of first strand cDNA amplified in a 40 μL reaction volume. The PCR reaction mixture contained: 1X Pfu DNA polymerase buffer, 0.5 μM of each primer, 200 μM of each dNTP, and 1.25 U Pfu DNA polymerase (Stratagene Ltd., Cambridge, UK). Amplification conditions included an initial denaturation of 5 min at 94 °C, followed by 20 cycles each of 45 sec at 58 °C, 3 min at 72°C, and 1 min at 94 °C. Two primer sets (Table 3.1) were used in the PCR screening (MM2FP/GBSSR3 and GBSSF2/MMRTRP1). A 2 μL aliquot of the first PCR amplifications were used to conduct a second set of PCR amplifications as described above. Reaction mixtures lacking cDNA were used as controls in both sets of PCR amplification reactions.

Products from the second set of PCR amplification reactions were separated by electrophoresis on a 1.5 % agarose gel containing ethidium bromide , using 1 or 40 μL of the reaction per lane, and visualized with UV light. An agarose slice containing DNA of interest, ranging from 1 to 1.6 kb in size was cut from the gel. DNA fragments were purified from the agarose by centrifugation through siliconized glass wool at 16 000 x g for 30 min. A 3 μL aliquot of the eluted DNA fragments was added to a PCR reaction

Fig. 4.1 Generalized schematic diagram of the *Wx* genes of hexaploid wheat showing the position of RT-PCR primer sets MM2FP/GBSSR3 and GBSSF2/MMRTRP1. Exons are represented by boxes and introns are shown as lines. Abbreviations for specific sequences are as follows: translation start codon, ATG; codon encoding the first N-terminal amino acid of the mature Wx protein, produced after the cleavage of the transit peptide, GCC; and translation stop codon, TGA.

(15 μL final volume) mixture containing: 1X Taq DNA polymerase buffer, 200 μM of dATP, and 2.5 U Taq DNA polymerase (Boeh. Mann.). The PCR reaction was incubated at 72 °C for 4 h. The extension at 72 °C using Taq DNA polymerase was used to facilitate the cloning of the blunt-ended PCR fragments, derived using Pfu DNA polymerase, into the T-tailed vector (Marchuk *et al.*, 1991) of the Original TA Cloning Kit (Invitrogen, Carlsbad, CA).

4.3.5 DNA Sequencing

DNA sequencing reactions were performed using the Applied Biosystems Prism Dye-deoxy Cycle Sequencing Kit and run on an Applied Biosystems Prism 373 DNA sequencer (Perkin-Elmer, Markham, ON). Sequence was initiated from known vector sequence (pCR2.1; Invitrogen). Based on these runs, primers specific to the wheat *Wx* cDNA clones were constructed to extend the DNA sequence (Table 3.1). Each strand was completely sequenced and the entire sequence of each cDNA was confirmed on the opposite strand. DNA and deduced protein sequences were examined with the MEGALIGN program (Lasergene Biocomputing software DNASTAR) using a PAM 250 residue weight table. Sequences were compared using the Cluster method with pairwise alignment parameters set at a K-tuple of 1 and gap penalty of 3 and with multiple alignment parameters set at a gap penalty and gap length penalty of 10.

4.4 Results and Discussion

4.4.1 Characterization of Waxy Protein Profiles and Amylose Concentrations

The wheat lines were characterized as possessing distinct Wx protein profiles (Fig. 4.2) and normal, intermediate or low amylose concentrations (Table 4.1). CDC Teal, the control line, possessed a wildtype amylose concentration. The F_6 progeny lines, CDC Wx2 and CDC Wx6, possessed distinct Wx protein profiles relative to their parental lines Kanto 107, lacking Wx-A1 and Wx-B1 proteins, and Bai-Huo, lacking the Wx-D1 protein. The waxy protein profiles of both parental lines were originally characterized by Yamamori *et al.* (1994). CDC Wx2 lacked all three Wx proteins and CDC Wx6 lacked Wx-D1 and Wx-B1 proteins. The parental line Bai-Huo had wildtype amylose

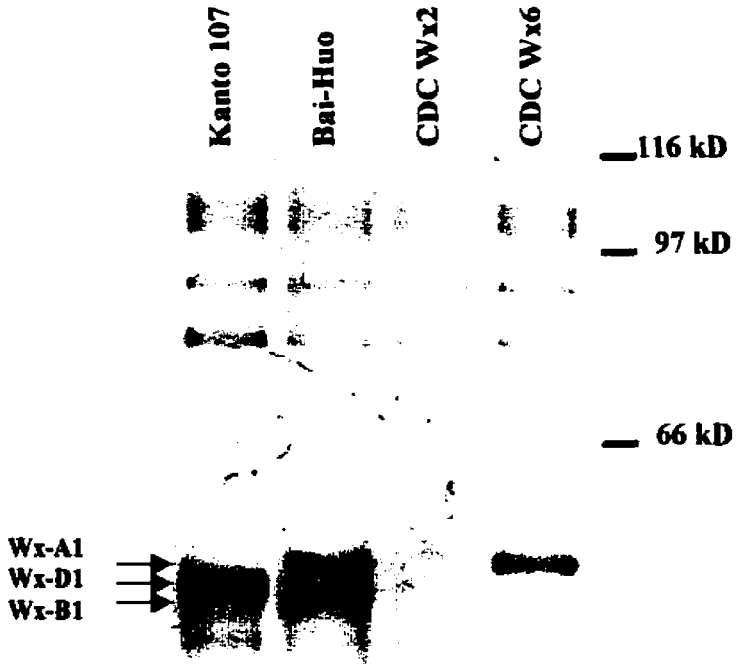


Fig. 4.2 Polyacrylamide gel electrophoresis separation of the Wx proteins (~ 59 kD) from wheat endosperm starch of Kanto 107 (*Wx-A1b Wx-B1b Wx-D1a*), Bai-Huo (*Wx-A1a Wx-B1a Wx-D1b*), CDC Wx2 (*Wx-A1b Wx-B1b Wx-D1b*), and CDC Wx6 (*Wx-A1a Wx-B1b Wx-D1b*). Gene products of the three wheat *Wx* loci are indicated on the gel. The migration distances of molecular weight markers are designated on the right.

Table 4.1 Amylose concentrations of the five wheat lines used in the study.

Line	Waxy protein phenotype†			Amylose concentration (%) ‡
	Wx-A1	Wx-B1	Wx-D1	
CDC Teal	+	+	+	26 (± 1) a
Bai-Huo	+	+	-	24 (± 2) a
Kanto 107	-	-	+	16 (± 1) b
CDC Wx6	+	-	-	14 (± 2) b
CDC Wx2	-	-	-	4 (± 1) c

† Presence (+) or absence (-) of waxy protein for each wheat line.

‡ Values are based on triplicate determinations.

a-c Means followed by the same letter are not significantly different (p=0.05), based on unpaired t-tests.

Numbers in parentheses are standard errors.

concentration when compared to CDC Teal. Both the parental line Kanto 107 and CDC Wx6 exhibited no significant differences in their intermediate amylose concentrations. CDC Wx2 possessed a low amylose concentration. Using high-performance size-exclusion chromatography to separate de-branched starch samples, the waxy phenotype of CDC Wx2 and partially waxy phenotype of CDC Wx6 have been associated with the reduced amylose concentrations of 0 % and 13 %, respectively (Demeke *et al.*, 1999). The Megazyme procedure described by Gibson *et al.* (1997) uses Con A to complex and selectively precipitate amylopectin. Demeke *et al.* (1999) compared amylose determination procedures and reported that the waxy CDC Wx2 line had an amylose concentration of 6 % using the Megazyme procedure and an amylose concentration of 0 % using high-performance size-exclusion chromatography. These researchers suggested that the amylopectin in the waxy line might be less branched thereby possessing a lower molecular weight. This lower molecular weight amylopectin polymer was assumed to be inefficiently precipitated by Con A in the Megazyme procedure.

4.4.2 Characterization of Waxy Transcript Levels in CDC Wx2 and CDC Teal

The expression of a 2.4 kb *Wx* transcript has been shown to accumulate throughout grain filling to the highest levels at 10, 15, and 20 DPA in wildtype hexaploid Chinese Spring wheat (Ainsworth *et al.*, 1993). The waxy phenotype of CDC Wx2 (three null *Wx* alleles) is associated with reduced levels of a 2.4 kb *Wx* transcript when compared to CDC Teal, the wildtype control (Fig. 4.3). Vrinten *et al.* (1999) also detected a very faint hybridizing band in total RNA from kernels (10 DPA) of a waxy line and Kanto 107 (null *Wx-A1b* and *Wx-B1b* alleles) relative to Bai-Huo (null *Wx-D1b* allele) and a wildtype Chinese Spring line. Waxy transcript levels in Bai-Huo and Chinese Spring were comparable. As reported by Vrinten *et al.* (1999), our RNA blot results using total RNA indicate that *Wx* transcripts of similar molecular weight are detectable in both the waxy and wildtype control lines. We speculated that at least a portion of the 2.4 kb *Wx* transcripts detected in CDC Wx2 were poly(A)+ RNA transcripts. Therefore, the screening for aberrations (i.e., small insertions or deletions) in low abundance *Wx* mRNA transcripts was undertaken as outlined in the following section using RT-PCR analysis.

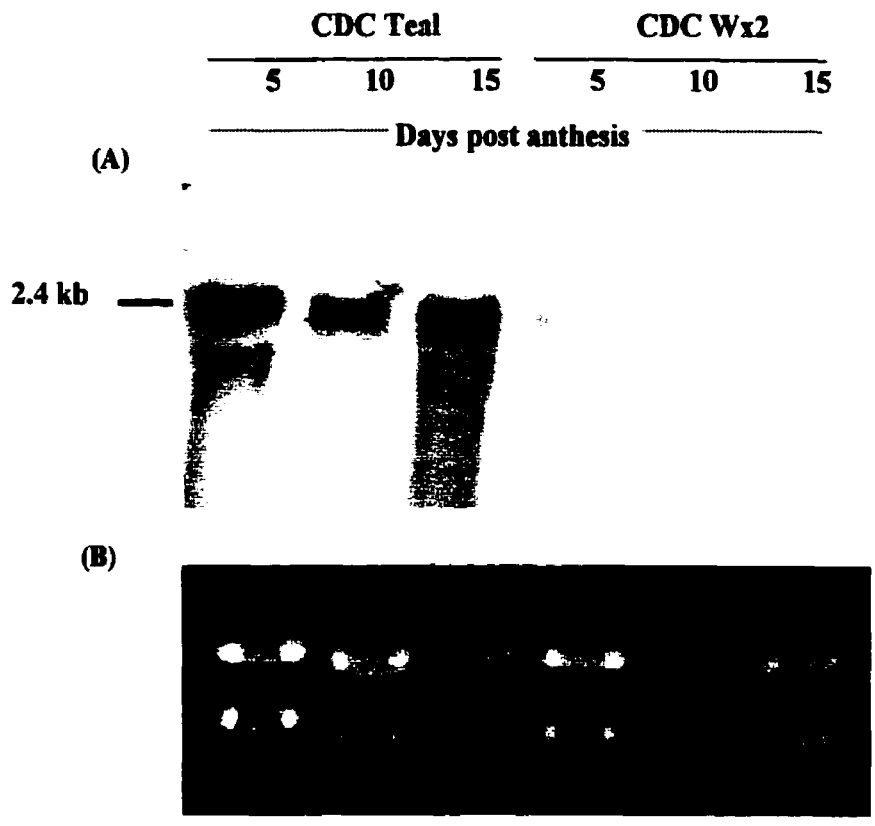


Fig. 4.3 Northern blot analysis of *Wx* gene expression in endosperm tissue of wheat. (A) RNA analysis performed with 5 μg total RNA. RNA bands are about 2.4 kb in size. (B) The ethidium bromide stained gel has been shown below the Northern blot to demonstrate that an approximately equal amount of total RNA was loaded across lanes. The migration distances of molecular weight markers are designated on the left.

4.4.3 cDNA Clones Detected Using Reverse Transcriptase-PCR

Reverse transcriptase-PCR analysis was conducted on CDC Wx2 and CDC Wx6 to detect aberrations within *Wx* mRNA transcripts. Parental lines were assayed to determine the parental origin of any aberrations. CDC Teal was included as a positive control. Using MM2FP and GBSSR3 primers, PCR amplification products of the expected 1383 bp size were detected in CDC Teal, Bai-Huo, and CDC Wx6 after 1 μL of the second PCR reaction was analyzed on a gel (Fig. 4.4A). PCR amplification products of the expected 1383 bp size were detected in Kanto 107, but were difficult to observe even after 40 μL of the second PCR reaction was analyzed on a gel (Fig. 4.4B). Two other PCR amplification products, both larger than the expected 1383 bp, were detected in CDC Wx2 and Kanto 107 (Fig. 4.4B). These aberrant PCR amplification products included a 1446 bp product and a relatively less abundant 1571 bp product. Ten cDNA clones from each of the five lines were randomly isolated and sequenced.

The relative frequencies of the three types of *Wx* transcripts (1383, 1446, or 1571 bp) detected within the lines are summarized in Table 4.2. The DNA sequences of the 1383 bp clones of CDC Teal (clone denoted Teal9-2), Bai-Huo (clone BH3-3), CDC Wx6 (clone Wx6-1), and Kanto 107 (clone K4-9) contained no aberrations relative to wildtype *Wx-A1a*, *Wx-B1a*, or *Wx-D1a* genomic sequences (Murai *et al.*, 1999; Appendix 7.26). DNA sequence alignments of the clones in this study and the wildtype *Wx* genomic sequences indicated that Teal9-2 and BH3-3 were encoded by the *Wx-B1* gene, K4-9 by the *Wx-D1* gene, and Wx6-1 by the *Wx-A1* gene. The DNA sequences of the 1446 bp clones detected in Kanto 107 (clone K4-2) and CDC Wx2 (clone K4-2) were 100 % identical (Table 4.2). The DNA sequences of the 1571 bp clones detected in Kanto 107 (clone K4-5) and CDC Wx2 (clone K4-5) were 100 % identical. Clone K4-2 contained one intron (66 nt), denoted intron 1 in this study. Clone K4-5 contained two introns, including intron 1 and another intron (125 nt) denoted intron 4 in this study. DNA sequences of clones K4-2 and K4-5 were 100 % identical, except for the presence of intron 4 in clone K4-5. Analysis of CDC Wx6 lacking Wx-B1 and Wx-D1 proteins failed to reveal aberrant *Wx* transcripts, suggesting that the RNA defects in this study are not responsible for the lack of the Wx-B1 or Wx-D1 proteins. DNA sequence alignments

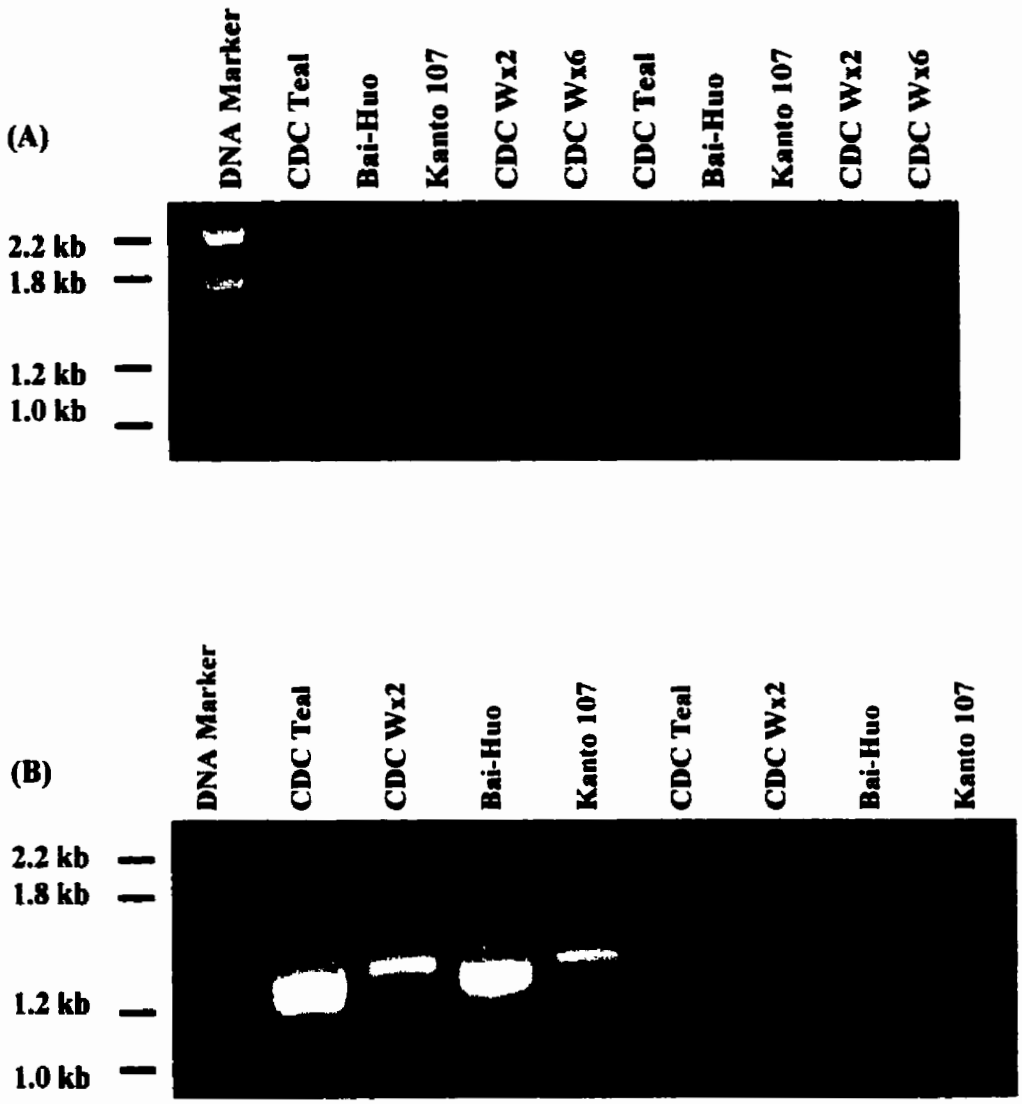


Fig. 4.4 RT-PCR amplification of *Wx* cDNA from wheat kernel total RNA. The gel analysis of PCR amplification products obtained using primers MM2FP and GBSSR3. (A) Second PCR amplification reactions (1 μL/lane) shown in lanes 2-6 contained cDNA and lanes 7-11 did not contain cDNA (no reverse transcriptase controls). (B) Second PCR amplification reactions (40 μL/lane) shown in lanes 2-5 contained cDNA and lanes 6-9 did not contain cDNA. The migration distances of molecular weight markers are designated on the left.

Table 4.2 Relative frequencies of three types of Wx transcripts detected within the lines.

Line	Wx protein phenotype †			Type of Wx Transcript ‡		
	Wx-A1	Wx-B1	Wx-D1	1383 bp	1446 bp	1571 bp
CDC Teal	+	+	+	10/10	0	0
Bai-Huo	+	+	-	10/10	0	0
CDC Wx6	+	-	-	10/10	0	0
Kanto 107	-	-	+	1/10	8/10	1/10
CDC Wx2	-	-	-	0	9/10	1/10

† Presence (+) or absence (-) of Wx protein in each wheat line.

‡ Wx transcripts containing no aberrations (1383 bp), intron 1 (1446 bp), and introns 1 & 4 (1571 bp).

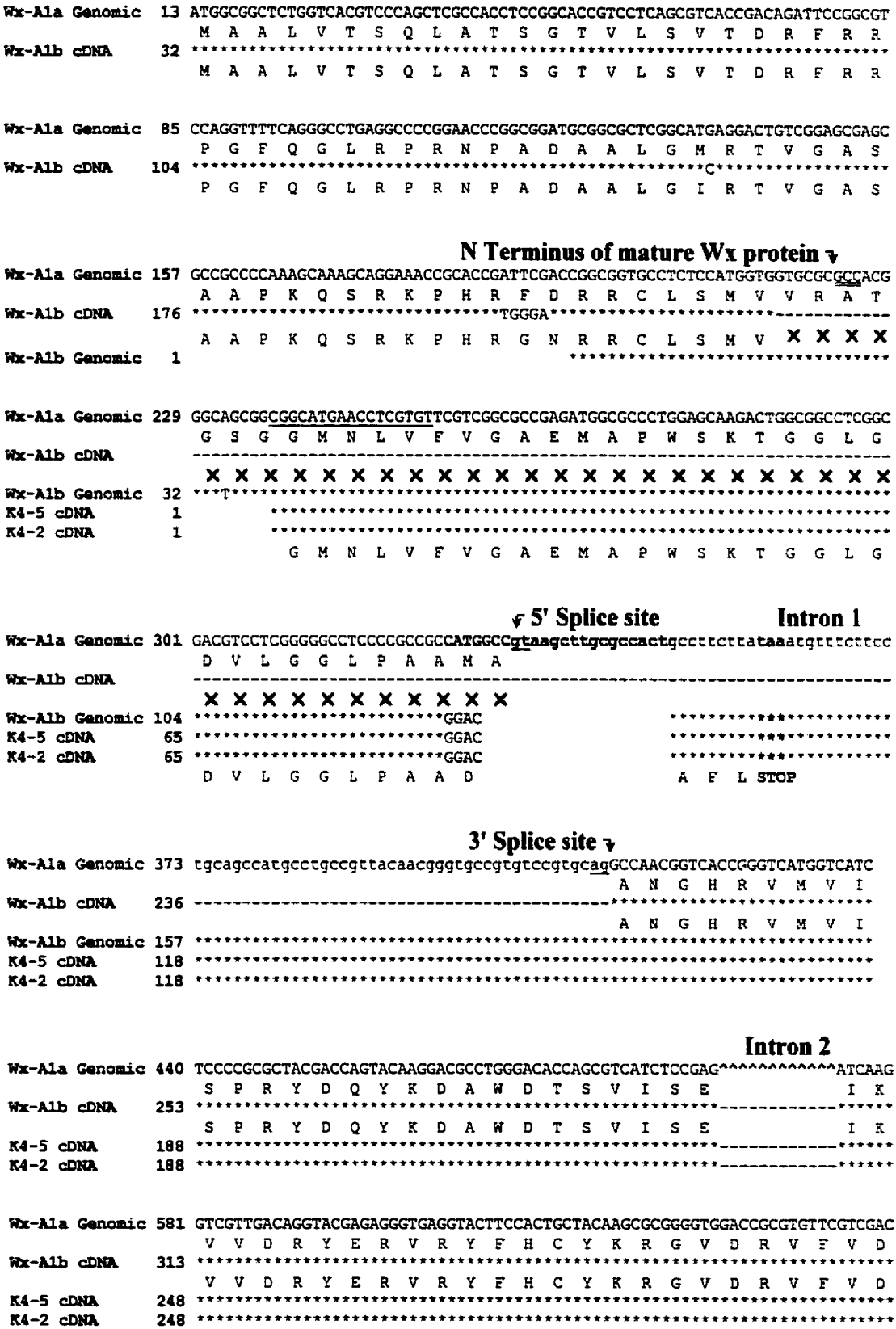
of the wildtype *Wx-A1a* genomic sequence (Murai *et al.*, 1999), the aberrant *Wx-A1b* cDNA (Vrinten *et al.*, 1999), the null *Wx-A1b* genomic sequence (Vrinten *et al.*, 1999), clones K4-5, and K4-2 indicated that K4-5 and K4-2 were encoded by the null *Wx-A1b* allele (Fig. 4.5).

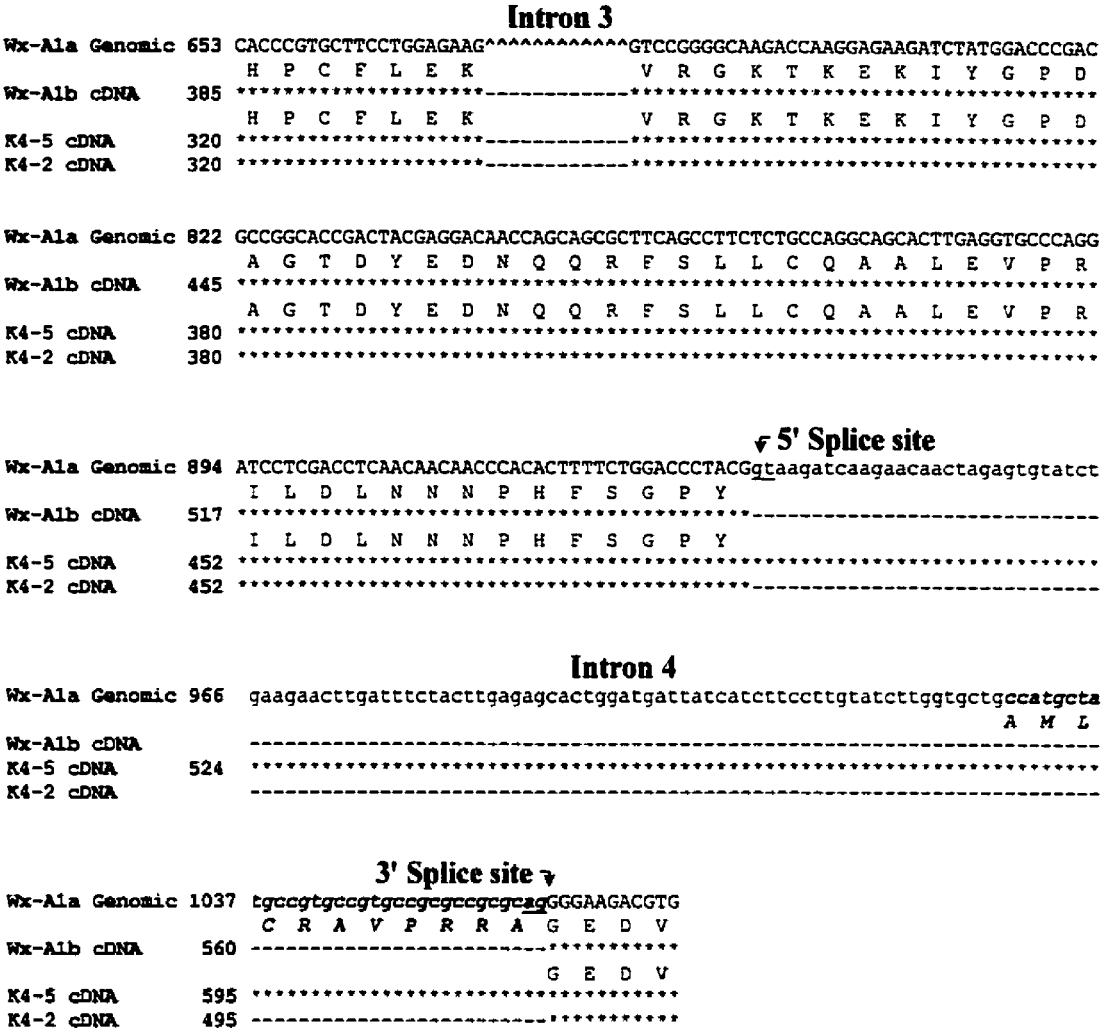
Using GBSSF2 and MMRTRP1 primers, only PCR amplification products of the expected size (1200 bp) were detected in Kanto 107, Bai-Huo, CDC Wx2, CDC Wx6, and CDC Teal (data not shown). Ten cDNA clones from each of the five lines were randomly isolated and sequenced. Clones from Kanto 107, Bai-Huo, CDC Wx2, CDC Wx6, and CDC Teal did not reveal any aberrations when compared to wildtype *Wx-A1a*, *Wx-B1a*, or *Wx-D1a* genomic sequences of wheat (Murai *et al.*, 1999; Appendix 7.27).

4.4.4 Deletion of 5' end of intron 1 Abolishes Normal Splicing

Vrinten *et al.* (1999) reported that the deletion of the normal 5' splice site of intron 1 has lead to the activation of at least one cryptic 5' splice site. The report described an aberrant *Wx-A1b* cDNA encoded by the null *Wx-A1b* allele of the waxy line which contained a 117 bp deletion (accession no. AF113843) when compared to a wildtype *Wx-A1a* genomic DNA sequence (Fig. 4.5). The 117 bp deletion resulted from the splicing of intron 1 through the use of a cryptic 5' GT splice site 117 bp upstream of the authentic 5' GT splice site identified in the wildtype *Wx-A1a* genomic DNA sequence. Similarly, splicing at a number of 5' cryptic splice sites has been detected and attributed to a GT to TT mutation at the 5' splice site of the first intron in the *waxy* gene of rice (Isshiki *et al.*, 1998). Several 5' cryptic splice sites were available upstream of the deleted 5' splice site of intron 1; however, no other aberrant *Wx* transcripts were detected by Vrinten *et al.* (1999). Larkin and Park (1999) have demonstrated that the selection of cryptic 5' splice sites in rice *Wx* transcripts are affected by temperature. A 5' splice site -93 nt upstream from that used in high amylose varieties predominated at 18°C. At higher temperatures, 25 and 32°C, there was a utilization of a 5' splice site at -1 and a non-consensus site at +1. This research suggests that the utilization of cryptic 5' splice sites in wheat *Wx* transcripts other than those described by Vrinten et al. (1999) may be detected using differential temperature regimes. The aberrant *Wx-A1b* cDNA (Vrinten *et al.*,

Fig. 4.5 Aberrant *Wx* transcripts encoded by the null *Wx-A1b* allele of CDC Wx2 and Kanto 107. DNA sequence alignments of the wildtype *Wx-A1a* genomic sequence (Murai *et al.*, 1999), a *Wx-A1b* cDNA encoded by null *Wx-A1b* allele (Vrinten *et al.*, 1999), the null *Wx-A1b* genomic sequence (Vrinten *et al.*, 1999), clone K4-5, and clone K4-2. The numbers refer to nucleotide numbering as appearing in GenBank. Exons are in uppercase letters and introns are in lower case letters. Omitted sequences of introns 2 and 3 are indicated by the symbol (^). An asterisk (*) indicates nucleotides identical to the wildtype *Wx-A1a* genomic sequence. The GCC codon encoding the first N-terminal amino acid of the mature protein, produced after the cleavage of the transit peptide, is double underlined. Primer MM2FP is underlined. Hyphens indicate spliced sequences. The 23 bp deletion described in the null *Wx-A1b* genomic DNA sequence has been bolded in the wildtype *Wx-A1a* genomic DNA sequence. Deduced Wx-A1 proteins are presented underneath their respective DNA sequences. The 39 amino acids deleted in the Wx-A1 protein of the *Wx-A1b* cDNA (Vrinten *et al.*, 1999) are represented by the symbol 'X'. The premature stop codons (TAA) incorporated by the presence of intron 1 in clones K4-5 and K4-2 are bolded. The truncated Wx-A1 protein sequence encoded by clones K4-5 and K4-2 is shown below the latter sequence.


Wx-A1a Genomic 13 ATGGCGGCTCTGGTCACGTCCCAGCTCGCCACCTCCGGCACCGTCCTCAGCGTCACCGACAGATTCCGGCGT
M A A L V T S Q L A T S G T V L S V T D R F R R
Wx-A1b cDNA 32
M A A L V T S Q L A T S G T V L S V T D R F R R
Wx-A1a Genomic 85 CCAGGTTTTCAGGGCCTGAGGCCCCGGAACCCGGCGGATGCGGCGCTCGGCATGAGGACTGTCGGAGCGAGC
P G F Q G L R P R N P A D A A L G M R T V G A S
Wx-A1b cDNA 104
P G F Q G L R P R N P A D A A L G I R T V G A S
N Terminus of mature Wx protein
Wx-A1a Genomic 157 GCCGCCCCAAAGCAAAGCAGGAAACCGCACCGATTCGACCGGCGGTGCCTCTCCATGGTGGTGCGCGCCACG
A A P K Q S R K P H R F D R R C L S M V V R A T
Wx-A1b cDNA 176
A A P K Q S R K P H R G N R R C L S M V
Wx-A1b Genomic 1
Wx-A1a Genomic 229 GGCAGCGGCGGCATGAACCTCGTGTTCGTCGGCGCCGAGATGGCGCCCTGGAGCAAGACTGGCGGCCTCGGC
G S G G M N L V F V G A E M A P W S K T G G L G
Wx-A1b cDNA
Wx-A1b Genomic 32
K4-5 cDNA 1
K4-2 cDNA 1
G M N L V F V G A E M A P W S K T G G L G
5' Splice site
Intron 1
Wx-A1a Genomic 301 GACGTCCTCGGGGGCCTCCCCGCCGCCATGGCCgtaagcttgcgccactgccttcttataaatgtttcttcc
D V L G G L P A A M A
Wx-A1b cDNA
Wx-A1b Genomic 104
K4-5 cDNA 65
K4-2 cDNA 65
D V L G G L P A A D
A F L STOP
3' Splice site
Wx-A1a Genomic 373 tgcagccatgcctgccgttacaacgggtgccgtgtccgtgcagGCCAACGGTCACCGGGTCATGGTCATC
A N G H R V M V I
Wx-A1b cDNA 236
A N G H R V M V I
Wx-A1b Genomic 157
K4-5 cDNA 118
K4-2 cDNA 118
Intron 2
Wx-A1a Genomic 440 TCCCCGCGCTACGACCAGTACAAGGACGCCTGGGACACCAGCGTCATCTCCGAG^^^^^^^^^^^^ATCAAG
S P R Y D Q Y K D A W D T S V I S E I K
Wx-A1b cDNA 253
S P R Y D Q Y K D A W D T S V I S E I K
K4-5 cDNA 188
K4-2 cDNA 188
Wx-A1a Genomic 581 GTCGTTGACAGGTACGAGAGGGTGAGGTACTTCCACTGCTACAAGCGCGGGGGTGGACCGCGTGTTCGTCGAC
V V D R Y E R V R Y F H C Y K R G V D R V F V D
Wx-A1b cDNA 313
V V D R Y E R V R Y F H C Y K R G V D R V F V D
K4-5 cDNA 248
K4-2 cDNA 248


Intron 3
Wx-A1a Genomic 653 CACCCGTGCTTCCTGGAGAAG^^^^^^^^^^^^^GTCCGGGGCAAGACCAAGGAGAAGATCTATGGACCCGAC
H P C F L E K V R G K T K E K I Y G P D
Wx-A1b cDNA 385
H P C F L E K V R G K T K E K I Y G P D
K4-5 cDNA 320
K4-2 cDNA 320
Wx-A1a Genomic 822 GCCGGCACCGACTACGAGGACAACCAGCAGCGCTTCAGCCTTCTCTGCCAGGCAGCACTTGAGGTGCCCAGG
A G T D Y E D N Q Q R F S L L C Q A A L E V P R
Wx-A1b cDNA 445
A G T D Y E D N Q Q R F S L L C Q A A L E V P R
K4-5 cDNA 380
K4-2 cDNA 380
5' Splice site
Wx-A1a Genomic 894 ATCCTCGACCTCAACAACAACCCACACTTTTCTGGACCCTACGgtaagatcaagaacaactagagtgtatct
I L D L N N N P H F S G P Y
Wx-A1b cDNA 517
I L D L N N N P H F S G P Y
K4-5 cDNA 452
K4-2 cDNA 452
Intron 4
Wx-A1a Genomic 966 gaagaacttgatttctacttgagagcactggatgattatcatcttccttgtatcttggtgctgccatgcta
A M L
Wx-A1b cDNA
K4-5 cDNA 524
K4-2 cDNA
3' Splice site
Wx-A1a Genomic 1037 tgccgtgccgtgccgccgccgccagGGGAAGACGTG
C R A V P R R A G E D V
Wx-A1b cDNA 560
G E D V
K4-5 cDNA 595
K4-2 cDNA 495

1999) does not possess the region for binding primer MM2FP (Fig. 4.5). This explains why aberrant *Wx* transcripts carrying a 117 bp deletion were not detected in our study. Forward primer MM2FP is found 21 bp downstream of the cryptic 5' GT splice site described by Vrinten *et al.* (1999). No cryptic 5' splice sites are available within this 21 bp region. The 117 bp deletion in the aberrant *Wx-A1b* cDNA resulted in a 39 amino acid deletion in the resultant Wx-A1 protein. The presence of an aberrant Wx-A1 protein with an altered molecular weight (~63.3 kD) was not detected in the endosperm starch of the waxy line. Therefore, these researchers concluded that the aberrant *Wx-A1b* cDNA in the waxy line was either not translated or the reduced abundance of *Wx* transcripts detected by Northern blotting lead to the translation of undetectable protein levels. In mammals, alternatively spliced mRNA transcripts are generally thought to remain undegraded; however, there is some evidence that RNA splicing errors without generation of a premature stop codon are associated with low levels of processed mRNA transcripts (Carstens *et al.*, 1991; Pajunen *et al.*, 1998). Based on this evidence in mammals, Vrinten *et al.* (1999) suggested that the aberrant *Wx-A1b* cDNA encoding a 63.3 kD non-functional Wx-A1 protein may contribute to reduced *Wx* transcript levels in their waxy wheat line.

Our results are an extension of the characterization of the null *Wx-A1b* allele recently reported by Vrinten *et al.* (1999). The presence of introns in the aberrant *Wx-A1b* pre-mRNA transcripts detected in this study may play a critical role in the establishment of cytosolic *Wx-A1b* mRNA transcript levels in CDC Wx2 and Kanto 107. It is generally accepted that pre-mRNA transcripts must undergo post-transcriptional processing in the nucleus, including the addition of 5' cap, methylation, polyadenylation, and intron splicing, to produce mature mRNA transcripts for export from the nucleus into the cytoplasm (Farrell, 1993). The aberrant *Wx-A1b* pre-mRNA transcripts detected in our study contain one (Clone 4-2) or two introns (Clone K4-5; Fig. 4.5) and are expected to be unstable in the nucleus. Intron 4 (detectable at position 495 to 619) is inefficiently excised from clone K4-5 (1571 bp) to produce the relatively more abundant K4-2 clone (1446 bp). This splicing mechanism appears to use the normal 5' and 3' splice sites reported in the wildtype *Wx-A1* genomic sequence. Inefficient intron splicing has also

been reported for intron 9 of the *waxy* gene in maize (*Zea mays* L.; Goodall and Filipowicz, 1991). Both clones K4-2 and K4-5 contain the 23 bp deletion at the 5' end of the exon 1-intron 1 junction, and 4 bp DNA filler insertion (nucleotides GGAC), characterized in the null *Wx-A1b* genomic DNA sequence of a waxy line (Fig. 4.5). The 23 bp deletion eliminated the 5' splice site of intron 1 in the genomic DNA and thus clones K4-2 and K4-5 represent *Wx-A1b* transcripts, containing intron 1, encoded by the null *Wx-A1b* allele. Pre-mRNA transcripts containing unspliced introns are assumed to be unstable in the nucleus; however, extensive evidence for the instability or stability of pre-mRNA transcripts containing introns in plants is currently limited. Isshiki *et al.* (1998) reported the inefficient excision of intron 1 from the 5' untranslated region of the *Wx* transcript of waxy rice and associated the GT to TT mutation with reduced *Wx* transcript and Wx protein levels. They studied a *Wx* allele (Wx^b) that resulted in a 10-fold decrease in *Wx* mRNA and protein production when compared to a wildtype *Wx* (Wx^a) allele. As previously reported by other groups, the Wx^b allele had the same GT to TT mutation at the 5' splice site of intron 1 and resulted in alternative splicing at three cryptic 5' splice sites. They introduced single base mutations to the 5' splice sites of both the Wx^a (GT mutated to TT) and Wx^b (TT mutated to GT) alleles, fused them to the *GUS* reporter gene, and introduced them into rice protoplasts. Their results demonstrated that the low expression level of the Wx^b allele resulted from the single base mutation at the 5' splice site of intron 1. Therefore, based on our limited knowledge, the low abundance of *Wx-A1b* mRNA transcripts detected using RT-PCR analysis may be explained by the decreased formation of mature *Wx-A1b* mRNA transcripts in the nucleus due to inefficient intron splicing in CDC Wx2 and Kanto 107.

The presence of premature stop codons in the aberrant *Wx-A1b* mRNA transcripts detected in this study may also play a critical role in the establishment of cytosolic *Wx-A1b* mRNA transcript levels in CDC Wx2 and Kanto 107. Intron 1, detectable at position 95 to 160 in clones K4-2 and K4-5, contains a premature termination codon (TAA; Fig. 4.5). In fact, introns are generally known to have termination codons in all three reading frames (Farrell, 1993). The disruption of the open reading frame by intron 1 results in the production of a severely truncated Wx-A1 protein (~4 kD with the cleavage or ~11 kD

without the cleavage of its transit peptide), instead of a mature 59 kD Wx-A1 protein, in CDC Wx2 and Kanto 107. The insertion of a premature stop codon in the 5' region of a mature mRNA transcript is known to target the transcript for rapid decay in plants (Johnson *et al.*, 1998). Thus, the premature stop codons in these two aberrant *Wx* transcripts in CDC Wx2 may contribute to, at least a portion of, the *Wx* RNA transcript instability detected by Northern blot analysis in waxy line CDC Wx2 (Fig. 4.5). If the *Wx* transcripts containing premature stop codons in this study are exported from the nucleus to the cytoplasm, then the low accumulation of these aberrant *Wx* transcripts in the cytoplasm may lead to the translation of a truncated Wx-A1 protein. Unfortunately, it would be extremely difficult to differentiate the resultant truncated Wx-A1 protein in a gelatinized starch sample from the degraded protein products commonly observed on silver stained 1-D SDS polyacrylamide gels.

Future studies including nuclear runoff assays, in conjunction with Northern blot analyses, are required to determine whether the null *Wx-A1b* genes in CDC Wx2 and Kanto 107 are regulated at the transcriptional (variables influencing the efficiency and/or rate of transcription; Farrell, 1993) or post-transcriptional level. Post-transcriptional regulation is any event that influences the splicing of pre-mRNA, pre-mRNA stability in the nucleus, nucleocytoplasmic transport, or stability of the RNA molecules in the cytoplasm. If post-transcriptional regulation of the null *Wx-A1b* gene is implicated, then the differential influence, if any, of the aberrant *Wx-A1b* transcripts, in our study and of Vrinten *et al.* (1999), on *Wx* RNA transcript stability in the nucleus or cytoplasm can be better understood.

Clark *et al.* (1991) were the first to clone and sequence a *Wx* cDNA from hexaploid wheat (accession no. X57233; 60.1 kD Wx protein). Other reports later established that the *Wx* cDNA isolated by Clark *et al.* (1991) was encoded by the *Wx-A1* gene of hexaploid wheat (Vrinten *et al.*, 1999; Murai *et al.*, 1999). Ainsworth *et al.* (1993) were the first to identify that the only major difference between the Wx-A1 protein, derived from the *Wx* cDNA isolated by Clark *et al.* (1991), and other plant Wx proteins was an 11 amino acid insertion (AMLCRAVPRRA). The 3' terminal DNA sequence of intron 4 of the wildtype *Wx-A1a* genomic sequence encodes the exact same 11 amino acid insertion (bolded and

italicized in Fig. 4.5) described by Ainsworth *et al.* (1993). It is tempting to speculate that the *Wx-A1* gene encoding the *Wx* cDNA isolated by Clark *et al.* (1991) must have had an AG/, instead of a TG/, dinucleotide 33 bp upstream of the normal 3' splice site of intron 4. This change alters the position of the 3' splice site of intron 4 and results in a 33 bp DNA insertion into the resultant *Wx-A1* transcript; however, this single basepair change has yet to be characterized at the genomic DNA level in wheat (Murai *et al.*, 1999). The functional significance, if any, of this small insertion in the resultant Wx-A1 protein remains undetermined (Ainsworth *et al.*, 1993). This small insertion is likely the product of an allelic polymorphism corresponding to the *Wx-A1* locus of hexaploid wheat. Future studies including the site-directed mutagenesis of regions within or adjacent to intron 4 , for example the 5' or 3' splice sites, branch points, or GC nucleotide composition (Goodall and Filipowicz, 1991; Simpson and Filipowicz, 1996), are required to determine the exact source(s) of the inefficient splicing of intron 4 in the null *Wx-A1b* gene in CDC Wx2 and Kanto 107. Improving the match of the 3' splice site of maize waxy intron 9 to the 3' splice site consensus (TGCAG↓GT; arrow denotes intron-exon splice junction) of monocots improved the efficiency of their processing in maize protoplasts (Goodall and Filipowicz, 1991). These studies may include the single basepair change (TG to AG) discussed in this section to determine the affect of this change on the splicing efficiency of waxy intron 4.

4.5 Conclusions

- For CDC Wx2 (*Wx-A1b*, *Wx-B1b*, and *Wx-D1b* alleles), a waxy hexaploid wheat line, the results reported herein indicate that the absent Wx-A1 protein results from aberrant *Wx-A1b* transcripts possessing premature stop codons.
- CDC Wx2, with its reduced amylose concentration (4 %) and reduced *Wx* transcript (2.4 kb) level, was determined to have an unspliced intron (intron 1) in the abberant *Wx-A1b* transcripts.
- Unspliced intron 1 caused reduced *Wx-A1b* transcript stability by incorporating a premature termination codon into the open reading frame of the Wx-A1 protein.
- The resultant Wx-A1 protein product encoded by the aberrant *Wx-A1b* transcripts was a severely truncated Wx-A1 peptide (~4 or 11 kD) that would be interpreted as an absent ~59 kD Wx-A1 protein using standard SDS-PAGE screening protocols.
- These conclusions are based on the evaluation of parental lines Kanto 107 and Bai-Huo, and partially waxy sister line CDC Wx6 (*Wx-A1a*, *Wx-B1b*, and *Wx-D1b* alleles).

5.0 GENERAL DISCUSSION

This study has focused on granule-bound starch synthase I, commonly known as the Wx protein, which is responsible for amylose synthesis in wheat kernel starch. The major objectives of the project were (1) to isolate and characterize a wheat cDNA encoding a Wx protein and (2) to identify aberrant *Wx* transcripts encoded by the null *Wx-A1b* allele of CDC Wx2, a waxy hexaploid wheat line, which result in an absent Wx-A1 protein (~59 kD). A summary of major conclusions and future directions of research corresponding to each research objective are outlined below.

5.1 Isolation and Characterization of a Wheat cDNA Encoding a Waxy Protein

5.1.1 Conclusions

- For GBSSMMI, a 2.2 kb cDNA insert within pBluescript (Accession no. Y16340), the results reported herein indicate that the wheat clone encodes a 66.3 kD Wx peptide.
- The Wx peptide encoded by cDNA Y16340 showed 100 % sequence similarities with deduced null (Accession no. AF113844) and wildtype Wx-D1 peptides (Accession no. AB019624).
- Deduced Wx peptide N-terminal sequences, isoelectric points, molecular weights, and amino acid sequence similarities indicate that clone GBSSIMMI encodes a Wx-D1 peptide originating from the D genome of hexaploid wheat.
- Functional analysis assays in glycogen synthase deficient RH98 cells were unsuccessful in demonstrating that the over expressed Wx-D1 protein possessed biological activity.
- The biological activity of the Wx-D1 peptide encoded by GBSSIMMI should be assessed *in vivo* using a waxy wheat line.

5.1.2 Future Directions

The Wx wheat clone may be used to study (1) the biological activity of Wx proteins, expression of *Wx* transcripts, and regulation of *Wx* genes *in planta*, (2) the co-supression of endogenous *Wx* genes, (3) the antisense inhibition of *Wx* transcripts, (4) the inheritance and segregation of *Wx* transgenes, (5) the formation and deposition of amylose in transgenic wheat kernels, (6) the *in vivo* role of the Wx protein in amylopectin synthesis, (7) the functional analysis of Wx transit peptides, and (8) the structure and function of Wx proteins using mutagenesis analysis. These studies, if performed using the various soluble and granule-bound starch synthase isoforms, will contribute to a better understanding of the exact roles of starch synthase isoforms in the production of starch, in both non-photosynthetic and photosynthetic tissues.

5.2 Identification of Aberrant *Wx* Transcripts Encoded by the Null *Wx-A1b* Allele

5.2.1 Conclusions

- For CDC Wx2 (*Wx-A1b*, *Wx-B1b*, and *Wx-D1b* alleles), a waxy hexaploid wheat line. the results reported herein indicate that the absent Wx-A1 protein results from aberrant *Wx-A1b* transcripts possessing premature stop codons.
- CDC Wx2, with its reduced amylose concentration and reduced *Wx-A1b* transcript (2.4 kb) level, was determined to have an unspliced intron (intron 1) in the abberant *Wx* transcripts.
- Unspliced intron 1 caused reduced *Wx-A1b* transcript stability by incorporating a pre-mature termination codon into the open reading frame of the Wx-A1 protein.
- The resultant Wx-A1 protein product encoded by the aberrant *Wx-A1b* transcripts was a severely truncated Wx-A1 peptide (~4 or 11 kD) that would be interpreted as an absent ~59 kD Wx-A1 protein using standard SDS-PAGE screening protocols.
- These conclusions are based on the evaluation of parental lines Kanto 107 and Bai-Huo, and partially waxy sister line CDC Wx6 (*Wx-A1a*, *Wx-B1b*, and *Wx-D1b* alleles).

5.2.2 Future Directions

Future research is needed to determine if similar conclusions can be made for the null *Wx-A1b* alleles in other waxy or partially waxy lines in other genetic backgrounds. These additional studies will be useful in assessing the applicability of primers MM2FP/GBSSR3 in identifying wheat lines carrying a *Wx-A1b* null allele using a PCR-based screening assay. All current Australian soft wheat cultivars accepted for the udon noodle market have the null *Wx-B1b* allele (Panozzo and Eagles, 1998); however, the equivalent usefulness of incorporating null *Wx-A1b* or *Wx-D1b* alleles in these cultivars has not been addressed adequately in the literature. Future studies using all eight genotypes at *Wx* loci in common wheat, in different genetic backgrounds, will be useful in characterizing other *Wx* null alleles and in elucidating the relationships between *Wx* loci null allele genotypes and noodle production. PCR-based analysis using the methods outlined herein would likely be suitable for screening double-haploid derived lines due to the destruction of immature kernels at 10 DPA. The advantage of using a PCR-based analysis using primers MM2FP/GBBR3 on cDNA template derived from kernel specific mRNA at 10 DPA is centered around the use of immature kernels, instead of the embryo-less half of mature kernels; however, the technical difficulties of working with RNA template, and the expense of cDNA synthesis and PCR techniques will have to be weighed against current SDS-PAGE screening procedures. Lastly, the results presented herein may also contribute to studies of the pedigree of Kanto 107 and evolutionary studies of the *Wx* genes of wheat.

The studies outlined herein are pre-requisites for applied research such as the improvement of wheat grain quality through genetic engineering and the establishment of PCR-based assays to facilitate the screening of potential donor parents and advanced breeding lines through a plant breeding program. Applied research remains limited to the pace at which basic research elucidates and understands general problems important to agricultural research. Basic research is slowly accumulated and the questions under investigation must be addressed from various angles to ensure strong conclusions. The results presented herein will likely provide the basis for future research which will lead to a better understanding of the hexaploid genome of wheat and to applied applications for wheat crop improvement.

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7.0 APPENDICES

Appendix 7.1 Description of the Lambda ZAPII-cDNA Library:

cDNA libraries represent the information encoded in the messenger RNA (mRNA) of a particular tissue or organism. RNA molecules are exceptionally labile and difficult to amplify in their natural form. For this reason, the information encoded by the RNA is converted into a stable DNA duplex (cDNA) then inserted into a self-replicating lambda vector. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and examined with relative ease. A representative cDNA library should contain full-length copies of the original population of mRNA. cDNA libraries provide a method by which the transcription and processing of mRNA can be examined and interpreted to produce models for the flow of information responsible for the fundamental uniqueness of each tissue type and organism.

The ZAP-cDNA synthesis kit (Cat. no 200400, 200401, and 200402; Stratagene) method uses a hybrid oligo (dT) linker-primer, which contains an Xho I site (Fig. 7.1). First strand synthesis is primed with the linker-primer and is transcribed using reverse transcriptase (RT) and 5-methyl dCTP. The use of 5-methyl dCTP during first strand synthesis hemimethylates the cDNA, protecting it from digestion from certain restriction endonucleases such as Xho I. Therefore, on Xho I digestion of the cDNA, only the unmethylated site within the linker-primer will be cleaved.

First strand cDNA synthesis begins when RT, in the presence of nucleotides and buffers, finds a template and a primer. The template is mRNA and the primer is a 50 base oligonucleotide with the following sequence:

5' GAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTTTTTTTTTTTTTTT 3'

" GAGA" Sequence Xho I site Poly (dT)

This oligonucleotide was designed with a GAGA sequence to protect the Xho I restriction enzyme recognition site and an 18 base poly (dT) sequence. The restriction site allows the finished cDNA to be inserted into the Uni-ZAP XR vector in a sense orientation (Eco RI--Xho I) with respect to the lacZ promoter. The poly (dT) region binds to the 3' poly (A) region of the mRNA template, and RT begins to synthesize the first strand cDNA.

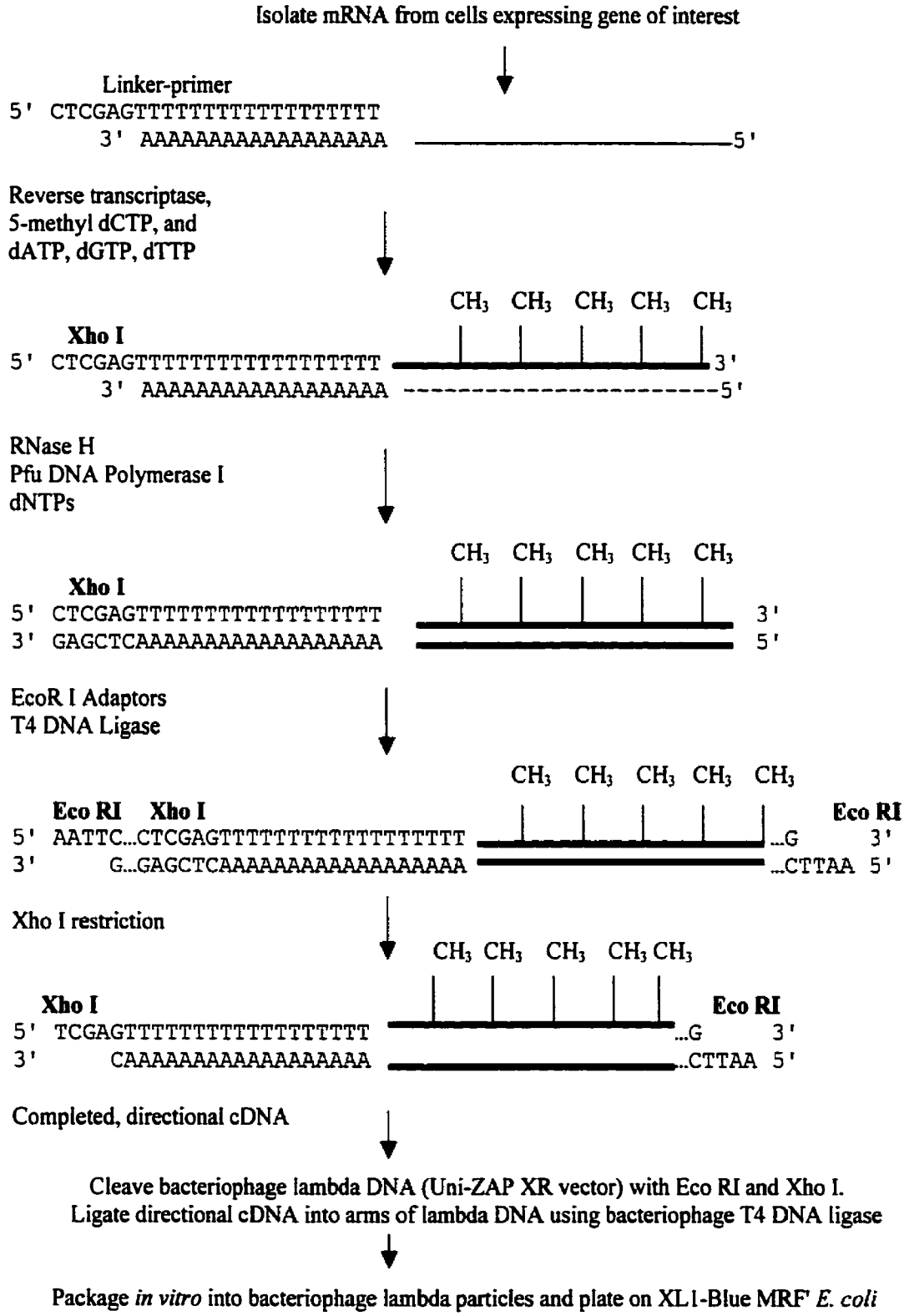


Fig. 7.1 ZAP-cDNA synthesis flow chart.

The nucleotide mixture for the first strand contains normal dATP, dGTP, and dTTP plus analog 5-methyl dCTP. The complete first strand will have a methyl group on each cytosine base which will protect the cDNA from restriction enzymes used in subsequent cloning steps.

During second strand synthesis, RNase H nicks the RNA bound to the first strand cDNA to produce a multitude of fragments, which serve as primers for Pfu DNA polymerase I. Pfu DNA polymerase I nick-translates these RNA fragments into second strand cDNA. The second strand nucleotide mixture has been supplemented with dCTP to reduce the probability of 5-methyl dCTP becoming incorporated in the second strand. This ensures that the restriction sites in the linker-primer will be susceptible to restriction enzyme digestion. The uneven termini of the double stranded cDNA are nibbled back or filled in with Klenow fragment (DNA polymerase with 3' to 5' exonuclease activity and no 5' to 3' exonuclease activity), and Eco RI adaptors are ligated (Ligases catalyze the repair of single-stranded nicks in duplex DNA and joins duplex restriction fragments having either blunt or cohesive ends.) to the blunt ends. The adaptors have the sequence shown below.

5' AATTCGGCACGAG 3'

3' GCCGTGCTC 5'

These adaptors are composed of 9 and 13 mer oligonucleotides, which are complimentary to each other with an Eco RI cohesive end. The 9 mer is kinased (Kinases catalyze the transfer of the terminal phosphate of ATP to the 5' hydroxyl termini of DNA and RNA.), which allows it to ligate to the other blunt termini available in the form of cDNA and other adaptors. The 13 mer is kept dephosphorylated (Phosphatases catalyze the hydrolysis of 5' phosphate residues from nucleic acids to produce dephosphorylated products with 5' hydroxyl termini.) to prevent it from ligating to other cohesive ends. After adaptor ligation is complete and the ligase has been heat inactivated, the 13 mer is kinased to enable its ligation into the dephosphorylated vector arms.

The Xho I digestion releases the Eco RI adaptor and residual linker-primer from the 3' end of the cDNA. These two fragments are separated on a Sephacryl column. The size-fractionated cDNA is then precipitated and ligated to the Uni-ZAP XR vector arms. The lambda library is packaged *in vitro* into bacteriophage lambda particles (Gigapack II Gold packaging extract; Cat no. 200402; Stratagene) and is plated on the *E. coli* cell line XL1-Blue MRF' (does not digest DNA containing 5'-methyl C). Fragments that have been cloned into the Uni-ZAP XR vector can be automatically excised from the bacteriophage lambda vector to generate sub-clones in the pBluescript SK (-) phagemid vector (vectors containing bacteriophage-derived origins of

replication), whose sequences are contained within the lambda DNA vector, eliminating the time involved in sub-cloning.

The Uni-ZAP XR vector system combines the high efficiency of lambda library construction and the convenience of a plasmid system with blue-white color selection. The Uni-ZAP XR vector is double digested with Eco RI and Xho I and will accommodate DNA inserts from 0 to 10 kb in length. The Uni-ZAP XR vector can be screened with DNA probes and allows *in vivo* excision of the Bluescript phagemid, allowing the insert to be characterized in a plasmid system. The polylinker of the Bluescript phagemid has 21 unique cloning sites by T3 and T7 promoters and a choice of 6 different primer sites for DNA sequencing. The phagemid has the bacteriophage f1 origin of replication, allowing rescue of single-stranded DNA.

The *E. coli* host strain XL1-Blue MRF' is supplied with the ZAP-cDNA synthesis kit. The F' episome present serves four purposes. (1) The F' episome contains the ΔM15 mutation of the lacZ gene required for alpha-complementation of the amino terminus of the lacZ gene present within the Uni-ZAP XR vector. (2) The F' episome contains the genes for expression of the bacterial F' pilli, which are required for filamentous phage infection. The conversion of a recombinant Uni-ZAP XR clone to a Bluescript phagemid requires superinfection with a filamentous helper phage (i.e., *in vivo* excision of the Bluescript phagemid from the Uni-ZAP XR vector). (3) The F' episome contains the lac repressor, which blocks transcription from the lacZ promoter in the absence of the inducer, isopropyl-B-D-thio-galactopyranoside (IPTG). (4) The tetracycline gene is also located on the F' episome in both strains; therefore in the presence of tetracycline, the episome is selectively maintained.

The ExAssist/SOLR system (Stratagene) is designed to allow excision of the Bluescript phagemid from the Uni-ZAP XR vector while preventing the problems that are associated with helper phage co-infection. The ExAssist helper phage contains an amber mutation that allows only the excised phagmid to replicate in the host, removing the possibility of co-infection from the ExAssist helper phage.

Appendix 7.2 Screening of the Lambda ZAPII-cDNA Library:

Library screening is outlined below and was conducted as described by the ZAP-cDNA Synthesis Kit (Cat. no 200400, 200401, and 200402; Stratagene) protocol:

❖ **Preparation of *E. coli* Host Cells:**

✓ The host strains are included in the kit as bacterial glycerol stocks. For the appropriate media and plates, please refer to the following:

Host Strain	Plates for bacterial streak	Media for bacterial cultures for titering phage (Final conc.)
SOLR	LB-kanamycin (50 µg/mL)	LB without a supplement
XL1-Blue MRF'	LB-tetracycline (12.5 µg/mL)	LB with 0.2 % (v/v) maltose-10 mM $MgSO_4$

✓ Revive the stored cells by scraping off solid ice with a sterile wire loop.

✓ Streak the splinters on to an LB plate containing the appropriate antibiotic (10 mg/mL kanamycin in water, aliquot, sterilize through a 0.22 micron filter, and store at - 20 °C in light-tight containers. A bacteriocidal agent (able to kill bacteria) that inhibits protein synthesis, translocation and elicits miscoding. 5 mg/mL tetracycline in methanol, aliquot, and store in light-tight containers without sterilizing. A bacteriostatic agent (inhibition of the proliferation of bacteria without killing them) the inhibits protein synthesis by preventing binding of aminoacyl tRNA to the ribosome A site.). Re-streak the cells each week.

✓ Prepare an -80°C bacterial glycerol stock as follows: In a sterile 50 mL tube inoculate 10 mL of appropriate liquid media containing antibiotic with one colony from the plate. Grow the cells to late log phase. Add 4.5 mL of a sterile glycerol liquid media solution (5 mL of glycerol and 5 mL of appropriate media) to the bacterial culture from step 1. Mix well. Aliquot into sterile centrifuge tubes (1 mL/tube). This preparation may be stored at -20°C for 1-2 years or at 80°C for more than 2 years.

❖ **Plating and Titering:**

✓ Check the titer of the library using host cells and serial dilutions of the library as follows:

- ✓ To plate the packaged ligation product, mix the following components in 2 mL tubes (each sample was done in duplicate): 1 µL of phage cDNA library and 200 µL of XL1-Blue MRF' cells at OD_{600}= 0.5; 1 µL of a 1:10 dilution of packaged reaction and 200 µL of cells; 1 µL of a 1:100 dilution of the library and 200 µL of cells; 1 µL of a 1:1000 dilution of the library and 200 µL of cells; 1 µL of a 1:10 000 dilution of the library and 200 µL of cells; and 1 µL of a 1:100 000 dilution of the library and 200 µL of cells. Further dilutions can be done if required.
- ✓ Incubate the phage and the bacteria at 37°C for 15 min to allow the phage to attach to the cells with shaking at 200 rpm.
- ✓ Add 15 uL of 0.5 M IPTG (in water) and 50 µL of 250 mg/mL X-gal (in demethylformamide) to 2-3 mL of NYZ top agar (48°C).
- ✓ Plate immediately onto the NYZ plates (spread agar by tilting plates from side to side) and allow the plates to set for 10 min. Place the plates upside down in a 37°C incubator.
- ✓ Plaques should be visible after 6-8 h. Plaques are a result of the phage infecting the bacteria, quickly multiplying within, and subsequent destruction (lysis) of their host cells.
- ✓ Determine the titer of the library (plaque forming units [pfu] per mL of library). Store plates at 4°C until needed.

❖ **cDNA Library Screening Protocol :**

- ✓ Titer library to determine concentration. Use fresh XL1-Blue MRF' *E. coli* host cells for titering and screening.
- ✓ Plate on large 150 mm NZY plates (> 2 days old; In 800 mL ddH_20 add 5 g NaCL, 2 g MgSO4 • 7 H_20, 5 g Yeast Extract, and 10 g NZ amine [Casein hydrolysate]. Adjust pH to 7.5 with NaOH. Add 15 g agar. Adjust volume to 1L. Autoclave. Allow the solution to cool to 55 °C. Pour ~ 80 mL/150 mm plate. Let harden, invert and store at 4°C.) to 50 000 pfu/plate with 600 µL of OD_{600} = 0.5 host cells per plate. (Use 20 plates to screen 1 x 10^6).
- ✓ Incubate phage and bacteria at 37 °C for 15 min with gentle shaking.
- ✓ Add 6.5 mL NZY top agar (In 800 mL ddH_20 add 5 g NaCL, 2 g MgSO4 • 7 H_20, 5 g Yeast Extract, and 10 g NZ amine [Casein hydrolysate]. Adjust pH to 7.5 with NaOH. Add 0.7 % (w/v) agar. Adjust volume to 1L. Autoclave. Maintain solution at 55°C.) to each phage/host cell mixture. Rub tube between palms to mix the mixture. Plate

immediately and distribute the top agar evenly over the plate by tilting the plate back and forth.

- ✓ Allow the plates to set for 10 min. Invert the plates and incubate at 37°C overnight. Plaques should be ~ 1 mm in diameter.
- ✓ Chill plates 2 h at 4°C to prevent top agar from sticking to Nylon membrane.
- ✓ Proceed with plaque lifts using Amersham Hybond-N+ nucleic transfer membranes.

❖ **Colony/Plaque Blotting:** (Based on a procedure provided with Amershan Hybond-N+ nucleic acid transfer membrane Cat. no. RPN137B [137 mm discs] and RPN82B [82 mm discs]).

- ✓ Use clean forceps and wear gloves during the entire procedure.
- ✓ Use a pencil to number each primary and duplicate lift.
- ✓ Transfer plaques onto a Hybond-N+ membrane (Cat. no. RPN137B) for 2 min. Use a clean needle to prick through the agar and mark the orientation of the lift.
- ✓ Place the membrane (DNA side up) on a clean filter paper. Allow the membrane to air dry.
- ✓ Make a duplicate lift by allowing the membrane to transfer for 4 min. Use a needle to mark the orientation of the lift.
- ✓ Store the plates at 4°C to isolate positive plaques after screening.
- ✓ Place the dry membranes (DNA side up) on filter papers soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 min.
- ✓ Transfer the membranes (DNA side up) to filter papers soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCL pH 7.2, 1 mM EDTA) for 3 min. Repeat once again with a fresh pad soaked in the same solution.
- ✓ Transfer membranes (DNA side up) to filter papers soaked in 0.2 M Tris-HCL pH 7.5 and 2X SSC buffer solution for 30 sec.
- ✓ Allow the membranes (DNA side up) to air dry 1 h on a 3MM paper and wrap in saran wrap. Fix sample to the membrane as described below.
- ✓ Crosslink DNA to membrane using autolink setting on a Stratalinker UV crosslinker (1.2 x 10^5 μJoules of UV light). Store at 4°C if unable to proceed with hybridization and detection as described in appendix 7.3.
- ✓ Store the NZY agar plates at 4°C for use after screening.

❖ **Secondary Library Screening:**

- ✓ Mark the positions of the needle pokes that were made through the membranes on to the film using a permanent marker.
- ✓ Identify the **strongest** putative clones which appeared on duplicate lifts.
- ✓ Orient the film and the stock plates using the marker dots on the film and the needle stabs through the agar.
- ✓ Putative clones were isolated from stock plates using an inverted 50 µL pipette tip and put into 1 mL SM buffer [5.8 g of NaCL, 2.0 g $MgSO_4 \cdot H_20$, 50.0 mL 1M Tris-HCL (pH 7.5), and 5.0 mL 2 % (w/v) gelatin] and 20 µL Chloroform. Vortex for 2 min. Store at 4°C.
- ✓ Dilute and titer with XL1-Blue MRF' *E. coli* host cells on small NZY plates so that one plate will have approximately 50 plaques. Perform serial dilutions of each putative clone sample with 300 µL of $OD_{600} = 0.5$ host cells per plate.
- ✓ Incubate phage and bacteria at 37°C for 15 min with gentle shaking.
- ✓ Add 3 mL NZY top agar to the phage/host cell mixture. Rub tube between palms to mix the mixture. Plate immediately and distribute the top agar evenly over the plate by tilting the plate back and forth.
- ✓ Allow the plates to set for 10 min. Invert the plates and incubate at 37°C overnight. Plaques should be ~ 1 mm in diameter.
- ✓ Chill plates 2 h at 4°C to prevent top agar from sticking to Nylon membrane.
- ✓ Proceed with plaque lifts using 82 mm Amersham Hybond-N+ nucleic transfer membranes (Cat. no. RPN82B) as described above and DIG hybridization and detection protocol as described in appendix 7.3.

❖ **Tertiary Screening:**

- ✓ Isolates may be picked from a secondary screen. If the positive plaques are too close to the background plaques then core, re-titer, and perform a tertiary screen to obtain the isolates.
- ✓ Follow the same procedure outlined in the secondary screening section above.
- ✓ Remember that phage diffuse in agar, so the screening procedure should be done quickly after lifts are taken. Once the plaque isolate has been cored from the plate and put in SM buffer with a drop of chloroform, it is stable at 4°C.

✓ When using the Uni-ZAP XR vector, automatic excision may be performed on the isolates to obtain the insert-containing Bluescript phagemid.

❖ ***In Vivo* Excision of the Bluescript Phagemid from the Uni-ZAP XR Vector:**

The Uni-ZAP XR vector has been designed to allow *in vivo* excision and re-circularization of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert. This excision is dependent on the DNA sequences that Startagene has placed in the lambda phage genome and on the presence of a variety of proteins, including f1 bacteriophage-derived proteins. The proteins from the f1 phage recognize a region of DNA, which normally serves as the f1 bacteriophage origin of replication for positive strand synthesis. However, the origin of the plus strand replication can be divided into two overlaying parts: (1) the site of initiation and (2) the site of termination for DNA synthesis. These two regions of the positive strand origin have been subcloned separately into the Uni-ZAP XR vector. The lambda phage (target) is made accessible to the f1-derived proteins by simultaneously infecting a strain of *E. coli* with both the lambda vector and the f1 bacteriophage. Inside *E. coli*, the helper proteins (i.e., proteins from f1 or M13 phage) recognize the initiator DNA that is within the lambda vector. These proteins then nick one of the two DNA strands. At the site of this nick, new DNA synthesis begins and duplicates whatever DNA exists in the lambda vector downstream of the nicking site. DNA synthesis of a new single strand of DNA continues through the cloned insert until a termination signal positioned 3' of the initiator signal is encountered within the constructed lambda vector. The single stranded DNA molecule is circularized by a gene II product from the f1 phage forming a circular DNA molecule, which contains everything between the initiator and terminator. In the case of the Uni-ZAP XR vector, this includes all sequences of the phagemid, Bluescript SK, (-), and the insert, if one is present. This conversion is a subcloning step, since all sequences associated with normal lambda vectors are positioned outside of the initiator and terminator signals and are not contained within the circularized DNA. In addition, the circularizing of the DNA automatically recreates a functional f1 origin as found in the f1 bacteriophage or phagemids.

Signals for packaging the newly created phagemid are contained within the f1 terminator origin DNA sequence. They permit the circularized DNA to be packaged and secreted from the *E. coli*. Once the phagemid is secreted, the *E. coli* cells used for *in vivo* excision of the cloned DNA can be removed from the supernatant by heating at 70°C. The heat treatment kills all the *E. coli* cells, while the phagemid remains resistant to the heat treatment. For production of double stranded DNA, the packaged Bluescript DNA is mixed with fresh *E. coli* cells and is spread on

LB ampicillin plates to produce colonies. DNA from mini-preps of these colonies can be used for analysis of insert DNA including sequencing, sub-cloning, and mapping.

The ExAssit/SOLR system is designed to allow efficient excision of the Bluescript phagemid from the Uni-ZAP vector, while eliminating problems associated with helper phage co-infection. The ExAssit helper phage contains an amber mutation that prevents replication of the phage genome in a nonsupressing strain such as SOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of productive co-infection from the ExAssist helper phage.

- ✓ Core plaque of interest from agar plate and transfer it to a sterile tube containing 500 uL of SM buffer and 20 uL of chloroform. Vortex the tube to release the phage particles into the buffer. Incubate 1-2 h at room temperature or overnight at 4°C. (This phage stock is stable for up to 1 year).
- ✓ Grow an overnight culture of XL1-Blue MRF' and SOLR cells in 25 mL of LB broth at 30°C.
- ✓ Make a 1:100 dilution of the cells grown overnight (50 uL) in 5 mL of LB broth. Grow at 37°C for 2-3 h to mid-log phase (OD_{600}=0.2-0.5).
- ✓ Gently spin down the XRL-Blue MRF' cells (1500 x g). Resuspend at OD_{600}=1.0 for single clone excision.
- ✓ Allow the SOLR cells to grow to OD_{600}=0.5-1. Before the SOLR cells reach OD_{600} greater than 1, remove the cells from the incubator and let them incubate at room temperature.
- ✓ In a 50 mL tube combine: 200uL of OD_{600}=1 XL1-Blue MRF' cells, 250 uL of phage stock (containing approx. 1 x 10^5 particles), and 1 uL of ExAssist helper phage (approx. 1 x 10^6 pfu/mL).
- ✓ Incubate mixture at 37°C for 15 min.
- ✓ Add 3 mL of LB broth and incubate for 2-2.5 h or overnight at 37°C with shaking.
- ✓ Spin down the cells for 15 min at 2000 x g. Transfer the supernatant to a fresh tube. Heat the tube at 70°C for 15 min and then spin again for 15 min at 4000 x g.
- ✓ Decant the supernatant into a sterile tube. This phage stock contains the excised phagemid Bluescript packaged as filamentous phage particles, and it can be stored at 4°C for 1-2 months.
- ✓ To plate the excised phagemids, add 200 uL of freshly grown SOLR cells (OD_{600} = 1) to two 1.5 mL tubes. Add 100 uL of the phage stock to one tube and 10 uL of the phage

stock to the other tube. Due to the high efficiency of the excision process, it may be necessary to titrate the supernatant to achieve single-colony isolation.

- ✓ Incubate tubes at 37°C for 15 min.
- ✓ Plate 10-50 uL from each tube on LB ampicillin plates (50 μg/mL) and incubate overnight at 37°C.
- ✓ Colonies appearing on the plate contain the Bluescript double stranded phagemid with the cloned DNA insert. Helper phage will not grow, since they are unable to replicate in the SOLR strain and do not contain ampicillin resistance genes. SOLR cells are also resistant to lambda phage infection, thus preventing lambda phage contamination after excision.
- ✓ To maintain the Bluescript phagemid, streak the colony on a new LB ampicillin plate. For long term storage, prepare a bacterial glycerol stock and store at -80°C.

❖ **Helper Phage Storage and Amplification:**

- ✓ Transfer a colony of XL1-Blue MRF' cells from a fresh LB tetracycline plate into 10 mL of 2X YT broth (10 g of NaCl, 10 g of yeast extract, and 16 g of bacto-tryptone; Adjust pH to NaOH; Autoclave) in a 50 mL tube.
- ✓ Incubate with shaking at 37°C until growth reaches OD_{600}=0.3.
- ✓ Add the ExAssist helper phage at a multiplicity of infection (MOI) of 20:1 (phage to cells).
- ✓ Grow at 37°C for 8 h.
- ✓ Heat at 65°C for 15 min.
- ✓ Spin down the cell debris and transfer the supernatant to a fresh tube.
- ✓ The titer of the supernatant should be between 7.5×10^{10} and 1.0×10^{12} pfu/mL.
- ✓ Add DMSO to a final concentration of 7 % and store at -80°C. If titer drops after 1 year of storage prepare a fresh high titer stock of the helper phage as outlined above.

Appendix 7.3 Digoxigenin-dUTP Hybridization and Detection Protocols:

The following hybridization and detection protocols are based on Engler-Blum *et al.* (1993):

❖ **Hybridization:**

- ✓ Wear gloves and use clean glassware and forceps throughout entire procedure.
- ✓ Turn on the hybridization oven and set at 68-65°C. Wash hybridization tubes with detergent and rinse with distilled water. Check the oven temperature by filling a hybridization tube with 25 mL of distilled water and inserting a thermometer into the tube.
- ✓ Roll membrane so that the fixed nucleic acids face toward the inside of the tube.
- ✓ Incubate membrane 1 h in pre-warmed pre-hybridization solution (0.25M Na_2HPO_4 pH 7.2, 1 mM EDTA, 20 % SDS) and 0.5 % blocking reagent (Blocking reagent dissolved in maleic acid buffer pH 7.5 [100 mM maleic acid, 150 mM NaCl pH 7.5] to give a milky suspension; Boeh. Mann.; Cat. no. 1096176) at 68-65°C with 20 mL/100 cm^2 with consistent redistribution of the solution.
- ✓ In a 500 µL tube, add 200 µL of sterile distilled water and 25 ng of DIG labeled cDNA probe (25 ng cDNA probe/10 mL of hybridization buffer). Boil the mixture for 10 min. Add the denatured probe to 10 mL pre-warmed hybridization solution (Pre-hybridization solution and 2.5 ng cDNA probe/mL).
- ✓ Replace the pre-hybridization solution with the hybridization solution containing 25 ng DIG-labeled cDNA probe.
- ✓ Incubate the filters overnight at 68-65°C with consistent redistribution of the solution. Probe solution can be re-used if stored at -20°C in a Falcon tube. For re-use, thaw, and denature by heating to 68°C for 10 min.
- ✓ Equilibrate 50 mL of pre-hybridization wash buffer (20 mM Na_2HPO_4, 1 mM EDTA, 1 % SDS) at 65-60°C. Replace hybridization solution with 50 mL of pre-warmed hybridization wash buffer. Wash for 20 min. Increase oven rotor speed to maximum for washing steps. Repeat step three more times.

❖ **Chemiluminescent Detection of DIG-Labeled Nucleic Acids:**

Using chemi-luminescent detection a light signal is produced on the site of the hybridized probe. The light signal can be recorded on X-ray films, requiring only very short exposure times. Chemi-luminescent detection is a three-step process. (1) Membranes are treated with blocking

reagent to prevent non-specific binding of antibody to the membrane. (2) Membranes are incubated with a dilution of anti-DIG, fab fragments, which are conjugated to alkaline phosphatase (AP). (3) The membrane carrying the hybridized probe and bound antibody conjugate is reacted with a chemi-luminescent substrate and exposed to X-ray film to record the chemi-luminescent signal.

- ✓ The volumes are calculated for a membrane size of 100 cm^2. Check pH of all solutions before proceeding with detection.
- ✓ In a clean tray, wash membranes 5 min in 25 mL detection wash buffer (0.1 M Maleic acid, 3M NaCl, 0.3 % Tween 20 pH 8.0) with shaking at room temperature.
- ✓ In another clean tray, incubate membranes for 1 h in 25 mL blocking buffer 2 (detection washing buffer, 0.5 % blocking reagent) with shaking at room temperature.
- ✓ Dilute 1.7 µL anti-Digoxigenin-AP conjugate (1:15 000; 0.75 U/µL; Cat. no. 1093274; Boeh. Mann.; Small antibody aggregates in the Anti-DIG-AP may lead to background in the detection. Centrifuge the vial for 5 min at 16 000 x g before the first use. After the first use it is sufficient to centrifuge the anti-DIG-AP for 1 min directly before dilution) in 25 mL blocking buffer 2 in a 50 mL falcon tube. Incubate membrane in a clean tray for 30 min with shaking at room temperature. (Fab fragments from an anti-DIG antibody from sheep, conjugated with alkaline phosphatase. After immunization with DIG the sheep IgG was purified by ion exchange chromatography and the specific IgG was isolated by immunosorption. The Fab fragments were isolated and conjugated with AP).
- ✓ Transfer membrane to a clean tray containing 50 mL of detection wash buffer to wash off unbound antibody-conjugate for 10 min. Repeat washing three more times using a clean tray each time.
- ✓ In a clean tray, equilibrate membrane for 5 min in 50 mL Substrate 4 buffer pH 9.5 (0.1 M Tris-HCL pH 9.5, 0.1 M NaCL, 50 mM MgCl) with shaking at room temperature.
- ✓ Prepare substrate solution 5 by diluting 0.2 mL CSPD (1:100; 25 mM Disodium 3-(4-methoxyspiro {1,2- dioxethane- 3,2'- (5' chlorotricyclo [3.3.1.1] decan}-4-yl) phenyl phosphate; Cat. no. 1655884; 11.6 mg/mL; Boeh. Mann.) in 20 mL substrate buffer 4 in a 50 mL Falcon tube. Incubate the membrane in a clean tray containing 20 mL substrate solution 5 for 5 min at room temperature with shaking. Dark conditions are not required. (CSPD is a chemi-luminescent substrate for alkaline phosphatase that enables extremely sensitive and fast detection of biomolecules by producing visible light which is recorded with film. Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to the metastable phenolate anion which decomposes and emits light at a maximum wavelength

of 477 nm. A delay in reaching maximum light emission results since the phenolate anion has a half-life of less than a minute to several hours, depending on the surrounding environment. The chemi-luminescent signal from CSPD persists for days on nylon membranes. Since film exposures of a few minutes are usually sufficient, multiple images may be acquired).

- ✓ Let the excess liquid drip off membrane and place membrane between a plastic sheet. Remove any air bubbles. Do not allow membrane to dry out since the membrane must be kept wet if it is to be re-probed.
- ✓ Tape the membrane enclosed within the plastic sheet into a cassette. Incubate the cassette at 37°C for 15 min to accelerate the chemi-luminescence reaction.
- ✓ Allow the cassette to incubate at room temperature for at least 2 h or overnight without x-ray film.
- ✓ Expose the membrane to x-ray film for 20 min. Re-expose if necessary. Luminescence continues for at least 24 h and signal intensity increases during the first hours.

Appendix 7.4 DNA Plasmid Isolation and Restriction Digestion Protocols:

For plasmid isolation and restriction analysis, colonies of interest were grown overnight in 2 mL LB broth containing 50 µg/mL ampicillin. The CTAB (Cetyl-tri-methyl-ammonium bromide) method of plasmid isolation described by del Sal *et al.* (1988) performed as described below. The strategy is based on the use of the cationic detergent CTAB for DNA precipitation.

- ✓ Transfer 1.5 mL of a 2 mL LB (50 µg/mL antibiotic) overnight bacterial culture to a 1.5 mL microfuge tube. Store the remaining culture volume at 4°C till restriction analysis is completed.
- ✓ Centrifuge at 16 000 x g for 2 min. Discard supernatant.
- ✓ Add 200 µL of STET (8 % Sucrose, 50 mM Tris-HCL, pH 8.0, 50 mM EDTA, and 0.1 % TritonX-100). Vortex tubes to resuspend cells.
- ✓ Add 4 µL of 50 mg/mL lysozyme (Sigma; Stored as 10 µL aliquots at -20°C). Incubate at 24°C for 5 min. Boil in a water bath for 45 sec.
- ✓ Centrifuge at 15 000 x g for 10 min. Discard pellet using a toothpick.
- ✓ Add 5 µL of 10 mg/mL RNase A (Sigma; Stored as 20 µL aliquots at -20°C). Incubate in a 68°C water bath for 10 min.
- ✓ Add 15 µL of 5 % (w/v) CTAB. Incubate at 24°C for 3 min.
- ✓ Centrifuge at 15 000 x g for 10 min. Discard supernatant.
- ✓ Gently resuspend pelleted DNA in 300 µL of 1.2 M NaCl.
- ✓ Add 750 µL of cold 95 % ethanol. Invert tube 6 times to mix. Centrifuge for 1 h at 4°C to pellet DNA. Remove supernatant using aspiration. The combination of salt and alcohol is used to concentrate the DNA. The DNA complexes with the monovalent cations in the solution and is recovered with the addition of ethanol and centrifugation.
- ✓ Add 1 mL of 80 % cold ethanol. Dislodge pellet by inverting and flicking the tube.
- ✓ Centrifuge and pellet DNA for 15 min at 4°C. Repeat 80 % ethanol wash two more times and then remove traces of ethanol using aspiration. Repeated washing of the DNA co-precipitate with diluted ethanol removes most of the salt used to drive the precipitation of the DNA.
- ✓ Vacuum-dry pelleted DNA for 5 min. Avoid over-drying pellet. Resuspend DNA in 25 µL of DNase-free water or TE buffer pH 7.4 (Tris-EDTA buffer: 10 mM Tris-CL pH 7.4, 1mM EDTA, pH 8.0). Incubate at 60°C for 10 min to promote resuspension of the DNA. Partially resuspended DNA samples have an A_{260}-A_{320}/A_{280}-A_{320} ratio < 1.6.

- ✓ Use 2 µL of resuspended DNA per 20 µL restriction digest reaction. Store the isolated plasmid DNA at 4°C until clones of interest have been identified using restriction analysis or sequencing.
- ✓ A 50 % (v/v) glycerol bacterial stock of clones of interest was prepared and stored at -80°C for long term storage.

To determine the concentration of the resuspended DNA, dilute the sample 100 fold by adding 1 µL of sample to 99 µL of TE buffer. Use TE buffer to blank the DU Series 7400 Beckman Spectrophotometer at 260 nm. Quantification of DNA in this fashion is predicted on the fact that nucleic acids absorb UV light maximally at 260 nm. Place the diluted sample into a 100 µL quartz cuvette and read the absorbance (in optical densities) at 260 nm (A_{260}), 320 nm (A_{320}), and 280 nm (A_{280}). Determine the DNA concentration by using the following formula:

$$[DNA]\ \mu g/\mu L = (A_{260} - A_{320}) \times 50 \times D \times 1\ mL/1000\ \mu L$$

where A_{320} is the absorbance at 320 nm and is used as a background correction (Neither proteins nor nucleic acids absorb at this wavelength, so absorbance is due to other components. Subtraction of the absorbance at any of the other wavelengths corrects for the background contribution); 50 is the extinction coefficient of DNA (an OD_{260} [optical density] of 1 corresponds to approximately 50 µg/mL of double stranded DNA); D is the dilution factor (D = 100 in the above example); and 1 mL/1000 µL converts the [DNA] from to µg/mL to µg/µL. Determine the DNA yield by multiplying the concentration by the volume of the DNA. Note that the A_{260} must be > 0.1 to give an accurate reading.

This calculation provides little information on the quality and purity of the sample, however in the presence of excess salt, contaminating proteins, and/or carryover organic solvents, the absorbance value can be skewed significantly. For these reasons, calculation of the 260:280 ratio (A_{260}-A_{320}/A_{280}-A_{320}) provides a reasonable estimate of the purity of the preparation. Absorbance at 280 nm is used for the detection of protein, based upon the presence of aromatic amino acids. A pure sample of DNA has a A_{260}/A_{280} ratio of 1.8 ● 0.05. If there is contamination with protein or phenol, the A_{260}/A_{280} ratio will be significantly less than the values given above, and accurate quantification of the amount of DNA will not be possible.

Restriction digests were performed in a 20 µL total reaction volume including 2U (one unit of enzyme is typically the enzyme activity that completely cleaves 1 µg of DNA under optimal reaction conditions) of restriction endonuclease (New England Biolabs, Boehringer Mannheim, or Pharmacia Biotech.) per µg of DNA and restriction reaction buffers provided by

the manufacturer. The reactions were incubated at the recommended temperature as suggested by the manufacturer for 1 h. Approximately 10 μL of the reaction volume was analyzed using an agarose gel of appropriate concentration to separate DNA fragments (Table 7.3). Restriction enzymes recognize and cut specific DNA sequences (e.g., Eco R1 [*Escherichia coli* RY13] restriction endonuclease recognizes the sequence 5'-G↓AATTC-3' and generates fragments with 5'-cohesive termini). Enzymatic reactions were terminated by heat inactivation at 85°C for 30 min.

Table 7.1 Agarose gel electrophoresis.

Agarose (%)	Size of DNA fragments separated (kb) †
0.7	1-30
1.0	0.5-10
1.2	0.4-7
1.5	0.2-3
2.0	0.05-2

† Bromophenol blue dye migrates approximately at 300 bp, and xylene cyanol dye at 4 kb, independent of agarose concentration between 0.5 and 1.4 % in 0.5X TBE.

Appendix 7.5 Criteria used to Design PCR Oligonucleotide Primers:

PCR oligonucleotide primers were designed using Primer Designer Version 2.0 (Scientific and Educational Software) to meet the following criteria (Innis *et al.*, 1990):

- Primer length ranged from 18 to 28 nucleotides.
- 50-60 % G and C nucleotide composition (greater composition values lead to primer hairpins and primer-dimers).
- Melting temperature (T_m= 2°C [A + T] + 4°C [G + C]; where the following nucleotides are represented: A, adenine; T, thymidine; G, guanidine; or C, cytosine) for the primer pair should be balanced (within 5°C) and between 55 and 80°C. An annealing temperature 5°C below the T_m of the amplification primer pair is typically used to optimize reactions. Annealing temperatures in the range of 55 to 72°C generally yield the best results in PCR reactions. Taq DNA polymerase is active over a broad range of temperatures thus primer extension will occur at low temperatures, including the annealing step. Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces mis-extension of incorrect nucleotides at the 3' end of primers. Caution must be taken to avoid T_m values lower than 55°C because lower annealing temperatures promote decreased PCR primer specificity.
- Avoid complementarity at the 3' ends of primer pair to avoid primer-dimers artifacts and reduction of yield of desired product.
- Avoid three or more C or G nucleotide at the 3' ends of primers to avoid mis-priming.
- Primers for mutagenesis contain mismatches placed internally for incorporating restriction enzyme sites. Mutagenic primers introduce specific experimental mutations. The following considerations should be made for designing mutagenic primers for use in the Quick Change Site-Directed Mutagenesis Kit described in appendix 7.12.
  - ✓ Both the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
  - ✓ Primers should be between 25 and 45 bases in length, and the melting temperature of the primers should be 10°C above the extension temperature of 68°C.
  - ✓ The desired mutation (deletion or insertion) should be in the middle of the primer 10-15 bases of correct sequence on both sides.
  - ✓ The primers optimally should have a minumum GC content of 40 % and should terminate in one or more C or G bases.

- ✓ Primers need not be 5' phosphorylated but must be purified either by fast polynucleotide liquid chromatography or by polyacrylamide gel electrophoresis.
- ✓ It is important to keep primer concentration in excess. Vary the amount of template while keeping the concentration of the primer constantly in excess.

Appendix 7.6 PCR Protocol using cDNA Library:

Most polymerase chain reaction (PCR) protocols use Taq DNA polymerase (EC 2.7.7). Taq DNA polymerase is isolated from the thermophilic eubacterium *Thermus aquaticus*, a strain lacking Taq I restriction endonulease and purified free of unspecific endo- or exo-nucleases. The enzyme consists of a single polypeptide chain with a molecular weight of ~ 95 kD. It is a highly processive 5'-3' DNA polymerase, that lacks 5'-3' and 3'-5' exonuclease activities. The enzyme exhibits the highest activity at pH 9 (adjusted at 20°C) and temperature around 75°C. Taq DNA polymerase activity is stable against prolonged incubations at elevated temperatures (95°C) and can therefore be used to amplify DNA-fragments by PCR. Taq DNA polymerase also accepts modified deoxyribonucleoside triphosphates as substrates, and can be used to label DNA-fragments either with radionucleotides or digoxigenin. The high processivity, absence of exo-nuclease activity and temperature optima of Taq DNA polymerase enable the use of this enzyme in DNA sequencing. PCR reactions using total phage cDNA library as the DNA template were prepared as follows:

- Prepare a reaction mixture on ice for the appropriate number of samples to be amplified. Add the components in order while mixing gently. Table 7.1 provides the reaction mixture for the amplification of PCR product from phage lysate. Bulk reaction mixture should be enough for the number of reactions plus an addition reaction volume. Mix the bulk reaction mixture well and briefly centrifuge. The final volume of each sample reaction is 25 µL.
- Immediately before thermal cycling, aliquot 20 µL of the bulk reaction mixture into the appropriate number of sterile thin-wall PCR tubes and place the tubes on ice.
- Overlay each reaction with two drops of mineral oil.
- Add 5 µL of phage lysate per reaction and gently stir reaction volume with pipette tip.
- Perform PCR using optimized cycling conditions as detailed in section 3.3.3.
- Analyze 10 µL of the PCR amplification products (resuspend to a final concentration of 1X with 6X loading buffer IV: 0.25 % bromophenol blue dye, 40 % (w/v) sucrose in water; Stored at 4°C) on a 100 mL 1X Tris-borate (TBE) 1.0 % (w/v) agarose gel (containing 0.2 µg/mL ethidium bromide) run at 100 V for 1 h to separate DNA fragments ranging from 0.5 to 10 kb in size.
- Ten µL of Boehringer-Mannheim Type VI DNA marker (50 ng/µL, including 11 DNA fragments ranging from 2.2 to 0.2 kb) and Type II DNA marker (50 ng/µL, including 8 DNA fragments ranging from 23 to 0.5 kb) were loaded to estimate the size of amplification products.

Table 7.2 Reaction mixture for the amplification of PCR products from phage lysate.

Component †	Amount per reaction
De-ionized water	11.1 µL
Taq DNA polymerase 10X reaction buffer ‡	2.5 µL
dNTPs (1.25 mM each NTP)	4.0 µL
GBSSF2 Primer (5 pmoles/µL) §	1 µL
GBSSR3 Primer (5 pmoles/µL)	1 µL
Phage lysate	5.0 µL
Taq DNA polymerase (5 U/µL) ¶	0.4 µL
Total reaction volume	25 µL

† All components stored at -20°C.

‡ 10 X reaction buffer: 0.1 M Tris-HCL (pH 8.8), 0.5 M KCL, 0.015 M $MgCl_2$, and 0.01 % gelatin.

§ Re-suspended in TE pH 8.0.

¶ Boehringer Mannheim; One unit Taq DNA polymerase is defined as the amount of enzyme that incorporates 10 nmol of total deoxyribonuclease-triphosphates into acid precipitable DNA within 30 min at 75°C under specific assay conditions.

Appendix 7.7 Original TA Cloning Kit:

The Original TA Cloning Kit with vector pCR2.1 provides a quick, one-step cloning strategy for the direct insertion of a PCR product into a plasmid vector. Taq DNA polymerase has non-template-dependent activity which adds deoxyadenosines (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. Thermostable polymerases containing extensive 3' to 5' exonuclease activity, such as Pfu DNA polymerase, do not leave 3' A-overhangs. PCR products generated with Taq polymerase have a high efficiency of cloning in the TA Cloning system as the 3' A-overhangs are not removed. If Pfu DNA polymerase is used, then 3' A-overhangs can be added to blunt-ended PCR fragments by incubation with Taq polymerase (10 min at 72 °C) at the end of the cycling program. The Original TA Cloning Kit is designed to facilitate the cloning of PCR fragments in a T-tailed plasmid vector known as pCR2.1 (3.9 kb). Important features found within the pCR2.1 vector are outlined in Table 7.2.

The Original TA Cloning protocol is briefly outlined as follows. (1) Generate the PCR product to be cloned into pCR2.1. (2) Modification of the PCR primer by phosphorylation or addition of a restriction site is not necessary. (3) The PCR product is ligated into pCR2.1 and transformed into competent cells. (4) PCR products are ligated into the vector in either orientation. Individual recombinant plasmids are analyzed by restriction mapping for orientation. (5) Recombinant plasmids of interest are purified for further sub-cloning or characterization.

❖ **Ligate PCR Products into pCR2.1 Vector:**

Amplify PCR products, the day prior to setting up the ligation reactions, as described in section 3.3.3. Use only fresh PCR product (less than one day old) because the single 3' A-overhangs on the PCR products will degrade over time, reducing ligation efficiency. The TA Cloning Reagents Kit (Invitrogen; Cat. no. K2000-01; Stored at -20°C) included 10X Ligation Buffer (60 mM Tris-HCL, pH 7.5, 60 mM $MgCl_2$, 50 mM NaCl, 1 mg/mL bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol, and 10 mM spermidine), pCR2.1 vector (25 ng/μL), and T4 DNA Ligase (4.0 U/μL). T4 DNA Ligase (EC 6.5.1.1) is isolated from an *E. coli* strain, which does not carry the gene coding for T4 RNA ligase. T4 DNA ligase catalyzes the formation of phosphodiester bonds between neighbouring 3' hydroxyl- and 5' phosphate ends in double stranded DNA. Single-stranded nicks in double stranded DNA are also closed by T4 DNA ligase. DNA fragments with over-lapping ends are incubated with T4 DNA ligase in 1X ligation buffer as follows:

Table 7.3 Features of pCR2.1 vector.

Feature	Function
lac promoter	For bacterial expression of the lacZα fragment for α-complementation (blue-white screening).
LacZα fragment	Encodes the first 146 amino acids of β-galactosidase. Complementation in *trans* with the Ω fragment gives active β-galactosidase for blue-white screening.
Ampicillin resistance	Selection and maintenance of *E. coli.*
ColE1 origin	Replication, maintenance, and high copy number in *E. coli.*
M13 Froward (-20 and -40) and M13 Reverse priming sites	Sequencing of insert.
Two unique Eco R1 sites within multiple cloning site/polylinker	T-tailed vector is designed for the inserted PCR product to be flanked on each side by Eco R1 sites.

- ✓ Briefly centrifuge one vial of pCR2.1.
- ✓ Set-up a 10 µL ligation reaction as follows: (Note: Do not use more than 2-3 µL of PCR sample in the reaction as T4 DNA ligase may be inhibited by salts in the PCR sample).

Components	Amount per reaction
Fresh PCR product	0.5-1.0 µL
10X Ligation Buffer	1 µL
pCR2.1 vector (25 ng/µL)	2 µL
T4 DNA Ligase (4 U/µL) †	1 µL
Sterile de-ionized water	Make up volume to 10 µL
Total reaction volume	10 µL

† 0.005 units T4 DNA ligase join more than 95 % of 1 µg Hind III digested lambda DNA in 20 µL 1X ligation buffer in 1 h at 22°C.

- ✓ Incubate at 14°C overnight.
- ✓ If transformation is not performed immediately then, store the ligation reaction at -20°C until needed.

❖ **Transform One Shot Competent Cells:**

At this point the ligation reaction contains the PCR insert ligated into the T-tailed vector. Transformations were done using the One Shot competent cell kit (Invitrogen; Cat. no. K2000-40) containing SOC Medium (2 % Tryptone, 0.5 % Yeast extract, 10 mM NaCL, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose), β-mercaptoethanol, and *E. coli* competent cells. Competent One Shot INVαF' cells were transformed as follows:

- ✓ Equilibrate a water bath to 42°C.
- ✓ Thaw 1 vial of SOC medium and bring to room temperature.
- ✓ Equilibrate Luria-Bertani (LB) plates (1.0 % Tryptone, 0.5 % yeast extract, 1.0 % NaCl pH 7.0, 1.5 % agar) containing 50 μg/mL ampicillin (50 mg/mL stock dissolved in water and filtered through a 0.22-micron filter. Aliquot and store at - 20°C in a light-tight container. Ampicillin is a bacteriocidal agent that kills only growing *E. coli*. The mode of action is to inhibit cell wall synthesis by inhibiting the formation of the peptidoglycan cross-link.) at 37°C for 30 min. Spread plates with 40 μL of 40 mg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Let the liquid soak in to plates 15 min. (To make a 40 mg/mL stock solution, dissolve 400 mg X-Gal in 10 mL dimethylformamide using a 15 mL Falcon tube. Protect from light by wrapping in aluminum foil. Store at -20°C.)
- ✓ Briefly centrifuge ligation reactions and place them on ice.
- ✓ Thaw on ice a vial of 0.5 M β-mercaptoethanol and one vial (50 μL) vial of frozen One Shot competent cells for each ligation/transformation reaction.
- ✓ Add 2 μL of β-mercaptoethanol to competent cell and mix by gently stirring with the pipette tip.
- ✓ Add 2 μL of ligation reaction to competent cells and mix by gently stirring with the pipette tip. Incubate vials on ice for 30 min. Store remaining ligation reaction mixtures at -20°C.
- ✓ Heat shock for 30 sec in a 42°C water bath without shaking. Immediately place vials on ice for 2 min.
- ✓ Add 250 μL of SOC medium to each vial.
- ✓ Incubate vials at 37°C for 60 min at 225 rpm. Place vials on ice.

- ✓ Spread 200 µL from each transformation vial on separate labeled LB plates containing antibiotic and X-Gal.
- ✓ Allow the liquid to absorb, invert plates and incubate them at 37°C at least 18 h. Transfer plates to a 4°C fridge for at least 4 h for proper color development.

❖ **Select Colonies for Plasmid Isolation for Restriction Digestion and DNA Sequencing**

For an insert size of 500 bp 50-200 colonies per plate will appear depending on the volume plated and of these about 80 % should be white on X-gal plates. Ligation efficiency depends on insert size. As insert size increases, the efficiency will decrease. To determine the presence and orientation of the insert:

- ✓ Pick at least 10 pure white colonies, without a blue center, for plasmid isolation and restriction analysis.
- ✓ Grow colonies overnight in 2-5 mL LB broth containing either 50 µg/mL of ampicillin.
- ✓ Isolate plasmid and confirm presence and orientation of cloned insert by restriction digestion and sequencing.

The pCR2.1 vector contains a piece of DNA that encodes an α fragment of β-galactosidase. This vector exploits the phenomenon called *α-complementation*. A cell that bears any of a number of deletions of the 5' end of the lacZ gene synthesizes an inactive C-terminal fragment of β-galactosidase, called an omega fragment. Similarly, a cell that bears a deletion of the 3' end of the lacZ encodes an inactive N-terminal fragment of β-galactosidase called an α fragment. However, if the cell contains two genes, one directing the synthesis of an α fragment, the other directing the synthesis of an omega fragment, the β-galactosidase activity is observed. The pCR2.1 vector incorporates a lac α fragment gene, which is small and easily manipulated. Exploitation of this vector requires use of a strain such as INVαF' *E. coli* cells carrying the complementing omega fragment gene to allow assembly of an active β-galactosidase complex. When INVαF' *E. coli* cells containing the pCR2.1 vector are grown on medium containing X-gal these vector containing cells possess β-galactosidase activity and turn blue. The substrate, X-gal, is turned blue by the enzymatic activity of β-galactosidase.

In order to make it easier to identify plasmids that contain insert DNA, the multiple cloning site of pCR2.1 has been engineered so that introduction of DNA into the polylinker results in a scorable phenotype. In INVαF' *E. coli* cells containing the pCR2.1 vector, production of the lacZ α fragment allows for formation of an active β-galactosidase enzyme which results in

the formation of blue colonies on X-gal indicator plates. Cloning into the polylinker of pCR2.1 prevents production of a functional lacZ α fragment, allowing for rapid identification of plasmid containing inserts as white colonies on X-gal plates incorporates a lac α fragment gene. The INVαF' *E. coli* cells do not express the lac repressor. Addition of the inducer IPTG (Isopropyl-1-thio-β-D-galactosidase) to X-gal indicator plates is not required. The inducer, IPTG, inactivates the lac repressor in cell expressing the lac repressor and thus de-represses omega peptide synthesis in the bacterial strain.

Appendix 7.8 PCR DIG Probe Synthesis Kit Protocol:

The non-radioactive digoxigenin (DIG) system uses DIG, a steroid hapten, to label DNA, RNA or oligonucleotides for hybridization and subsequent luminescence detection (Fig. 7.2). For DNA labeling, DIG is coupled to dUTP via an alkali-labile ester-bond. The use of the alkali-labile form of DIG-dUTP enables easier and more efficient stripping of blots for re-hybridization experiments with a second DIG-labeled probe. DNA probes, labeled with DIG-dUTP, alkali-labile cannot be denatured by alkali treatment (NaOH), but must be denatured by incubation in a boiling water bath.

The PCR DIG synthesis kit (Catalogue no. 1636 090; Boeh. Mann.; Stored at -20°C) contains all reagents for the direct DIG-labeling of DNA fragments generated by PCR. The PCR DIG mix supplied with the kit enables the synthesis of highly sensitive probes by incorporation of DIG-dUTP into the PCR product. The kit is designed for generation of sensitive hybridization probes suitable for detection of single copy target sequences. PCR products can directly be amplified and labeled from low amounts of genomic DNA (1-100 ng) or plasmid DNA (10-100 pg) and subsequently used as hybridization probes. The PCR DIG mix contains alkali-labile DIG-dUTP formulation. This enables simple removal of the DIG-label after chemi-luminescent detection and subsequent re-hybridization of blots with multiple DIG-labeled probes (Fig.7.3). The PCR labeling protocol was performed as follows:

❖ Add the following components to a sterile microfuge tube. Place the tube on ice during pipetting.

Components †	**Amount per reaction**
Distilled water	34.2 µL
Taq DNA polymerase 10X reaction buffer	5 µL
10X PCR DIG mix ‡	5 µL
GBSSF2 Primer (5 pmoles/µL)	2 µL
GBSSR3 Primer (5 pmoles/µL)	2 µL
Taq DNA polymerase (5 U/µL)	0.8 µL
GBSSIMMI clone template (50 pg/µL)	1 µL
Total reaction volume	50 µL

† All components stored at -20°C.

‡ Contains 2 mM each of dATP, dCTP, and dGTP; 1.3 mM dTTP; 0.7 mM DIG-11-dUTP; alkali-liable; pH 7.0.

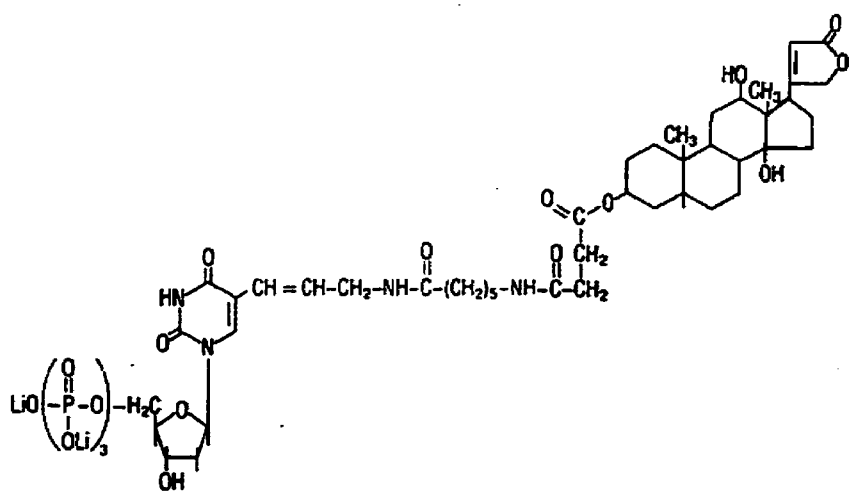


Fig. 7.2 Structure of digoxigenin-11-dUTP.

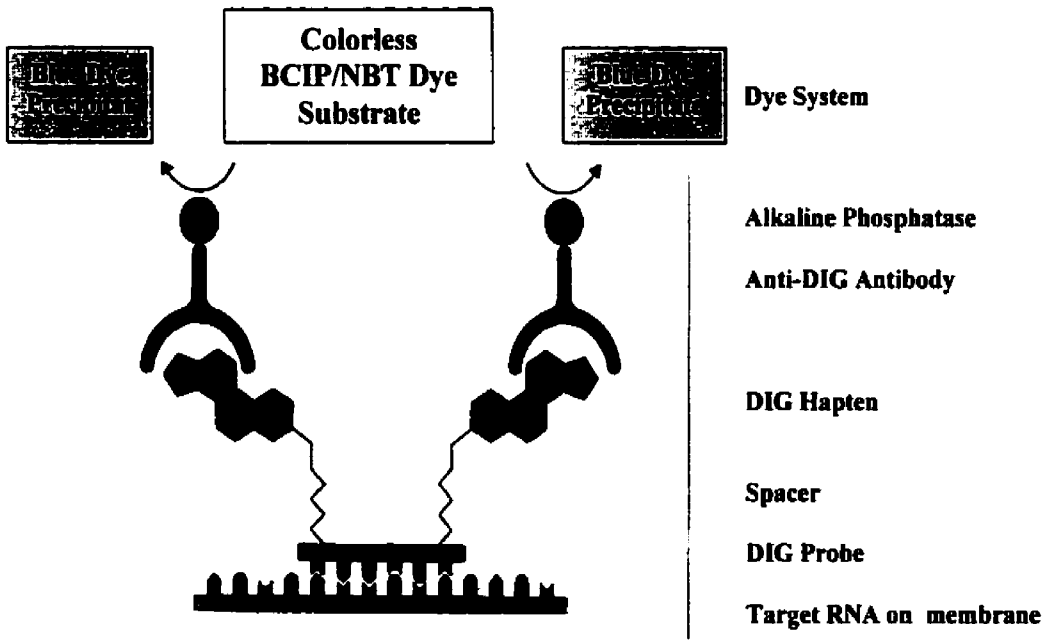


Fig. 7.3. Non-radioactive Northern blotting detection.

- Mix reagents and centrifuge briefly to collect the sample at the bottom of the tube.
- Overlay with two drops of mineral oil to reduce evaporation of the mix during amplification.
- Cycling conditions are as described in section 3.3.3.
- After amplification, analyze an aliquot of the reaction mixture (10 µL) by 1.0 (w/v) agarose gel electrophoresis. For use of the PCR product as a hybridization probe in blots, a specific band should be visible on an ethidium stained gel. Even minor amounts of by-products can influence the specificity of the hybridization. Optimal reaction conditions have been adapted, including incubation times, incubation temperatures, template concentration, and primer concentration. Due to the multiple incorporation of DIG-dUTP during the PCR process the molecular weight of the PCR product is increased significantly compared to the unlabeled product (prepare an unlabeled PCR product in parallel for better detection of the shift in molecular weight to evaluate the efficiency of DIG-dUTP incorporation into the probe). Each 50 uL labeling reaction typically yielded ~ 600 ng of DIG labeled probe (12 ng/µL). The quantity of DNA in the sample was estimated by comparing the florescent yield of the sample with that of a series of a standard (1, 5, 10, 20, and 30 µL of Boehringer Mannheim Type VI DNA marker [50 ng/µL], including 11 DNA fragments ranging from 2.2 to 0.2 kb). This [DNA] estimation method utilizes the UV-induced florescence emitted by ethidium bromide molecules intercalated into the DNA. As little as 1 to 5 ng of DNA can be detected by this method.
- The labeled PCR product was stored at 4°C and used directly without further purification in DIG hybridization and luminescent detection protocols as described in appendix 7.3.

Appendix 7.9 ABI PRISM Big Dye Terminator Cycle Sequencing Protocol:

Dideoxy DNA sequencing requires a clone of the DNA to be produced so that a homogeneous population of molecules is available for analysis. Preparation of template DNA for dideoxy sequencing involves several steps. (1) Consider a segment of DNA cloned into a restriction site of a plasmid cloning vector. The circular plasmid is denatured by heat to single strands. (2) An oligonucleotide primer, complimentary to the DNA insert of interest, is annealed to the DNA. The primer will anneal to only one of the two DNA strands. The oligonucleotide acts as a primer for DNA synthesis, and the 5' to 3' orientation chosen ensures that the DNA made is a complementary copy of the cloned DNA.

Dideoxy DNA sequencing of this theoretical DNA insert involves the following steps. (1) For each DNA fragment to be sequenced, one reaction is set up with single-stranded DNA to which the primer has been annealed. The reaction contains four normal precursors of DNA (dATP, dTTP, dCTP, and dGTP), dideoxy nucleotides (ddATP, ddTTP, ddCTP, and ddGTP), and DNA polymerase. The difference between the dideoxy nucleotide and the deoxynucleotides normally used in DNA synthesis is dideoxy nucleotide has 3'-H on the deoxyribose sugar rather than a 3'-OH. If a dideoxy nucleoside triphosphate (ddNTP) is used in the sequencing reaction, the dideoxy nucleotide can be incorporated into the growing chain. However, once that happens, no further DNA synthesis can then occur because the absence of a 3'-OH prevents the formation of a phosphodiester bond with an incoming DNA precursor. (2) In the reaction, only a small proportion of the precursors are dideoxy precursors (1:100 ratio ddNTPs:dNTPs). The primer is extended by DNA polymerase, and when a particular nucleotide is specified by the template strand, there is a small chance that the dideoxy nucleotide will be incorporated instead of the normal nucleotide in the reaction. For example, if an dNTP is specified by the template strand a ddNTP could be incorporated rather than dNTP in the reaction. Once a ddNTP is incoporated, elongation of the chain stops. In a population of molecules in the same DNA synthesis reaction, then, new DNA chains will stop at all possible positions where the nucleotide is required because of the incorporation of the dideoxy nucleotide. (3) The DNA chains in the reaction are separated by polyacrylamide gel electrophoresis and the DNA bands are read by a laser technology within the sequencing instrumentation. (4) The DNA bands can be detected by the laser because each of the four ddNTPs in the reaction are labeled with a different fluorescent dyes which emit maximum fluorescence at different wavelengths.

The ABI PRISM big dye terminator cycle sequencing ready reaction kit (Cat. no. 4303149; Perkin-Elmer) was used to sequence DNA inserts of interest within plasmid cloning vectors. The kit formulation contains the sequencing enzyme AmpliTaq DNA Polymerase. This

enzyme is a variant of *Thermus aquaticus* DNA polymerase that contains a point mutation in the active site. This results in less discrimination against dideoxynucleotides (ddNTPs), which leads to a much more even peak intensity pattern. Applied Biosystems (Perkin-Elmer) has developed a set of dye terminators labeled with novel, high-sensitivity dyes. The new dye structures contain a fluorescein donor dye, e.g., 6-carboxyfluorescein (6-FAM), linked to a dichlororhodamine (dRhodamine) acceptor dye. The excitation maximum of each dye label is that of the fluorescein donor, and the emission spectrum is that of the dRhodamine acceptor. The donor dye is optimized to absorb the excitation energy of the laser in the Applied Biosystems DNA sequencing instruments. The linker affords extremely efficient energy transfer (quantum efficiency nearly 1.0, i.e., 100 %) between the donor and acceptor dyes. The big dye terminators are 2–3 times brighter than the rhodamine dye terminators when incorporated into cycle sequencing products. The big dye terminators are labeled with the following dRhodamine acceptor dyes: A terminator, green dye dR6G; C terminator, red dye dROX; G terminator, blue dye dR110; and T terminator, yellow dye dTAMRA. Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the ABI PRISM 377 instruments, the software collects light intensities from four specific areas on the camera, each area corresponding to the emission wavelength of a particular fluorescent dye. The new big dye terminators also have narrower emission spectra than the rhodamine dye terminators, giving less spectral overlap and therefore less noise. The brighter signal and decreased noise provide an overall 4–5X improvement in sensitivity over older technologies.

The ABI PRISM big dye terminator cycle sequencing ready reaction kit combine AmpliTaq DNA polymerase, the new big dye terminators, and all the required components for the sequencing reaction. In the ready reaction format, the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, $MgCl_2$, and buffer are pre-mixed into a single tube of Ready Reaction Mix and are ready to use. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on double-stranded DNA templates. The dNTP mix includes dITP in place of dGTP to minimize band compressions. The dNTP mix also uses dUTP in place of dTTP. dUTP improves the incorporation of the T terminator and results in a better T pattern.

❖ **Preparing Sequencing Reactions:**

- ✓ The ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Store at – 25 °C) contain sufficient reagents to sequence 100 templates. Terminator Ready Reaction Mix contains: NTP mix (ddATP labeled with dichloro[R6G]; ddCTP labeled

with dichloro[ROX]; ddGTP labeled with dichloro[R110]; ddTTP labeled with dichloro[TAMRA]; dATP; dCTP; dITP; and dUTP); AmpliTaq DNA Polymerase, $MgCl_2$ (2 mM final conc. per reaction); and Tris-HCl buffer, pH 9.0.

- ✓ Sequencing primers were made using the criteria outlined in appendix 7.5.
- ✓ For each 20 µL reaction, add the following reagents to a separate tube: Terminator ready reaction mix 8.0 µL, double-stranded DNA template 200–500 ng; primer 3.2 pmoles; make up the final volume to 20 µL with de-ionized water.

✓ Mix well and spin briefly.

- ✓ Overlay the reaction mixture with 40 µL of light mineral oil.
- ✓ Place the tubes in a thermal cycler. Repeat the following for 25 cycles: 96 °C for 10 sec; 50 °C for 5 sec; 60 °C for 4 min.
- ✓ Rapid thermal ramp to 4 °C and hold until ready to purify.
- ✓ Spin down the contents of the tubes in a microcentrifuge.
- ✓ Proceed to purifying the sequencing products.

❖ **Spin Column Purification to Remove Excess Dye Terminators from Sequencing Products:**

- ✓ Gently tap the column (Centri-Sep spin columns from Princeton Separations; Cat. no. CS-901) to cause the gel material to settle to the bottom of the column.
- ✓ Remove the upper end cap and add 0.8 mL of de-ionized water.
- ✓ Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material.
- ✓ Allow the gel to hydrate at 24 °C for at least 2 h. Hydrated columns can be stored for a few days at 2–6 °C. Longer storage in water is not recommended. Allow columns that have been stored at 2–6 °C to warm to room temperature before use.
- ✓ Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.
- ✓ Remove the upper end cap first, then remove the bottom cap. Allow the column to drain completely by gravity. If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.
- ✓ Insert the column into the wash tube provided.
- ✓ Spin the column in a microcentrifuge at 730 x g for 2 min to remove the interstitial fluid.
- ✓ Remove the column from the wash tube and insert it into a 1.5 mL tube.
- ✓ Remove the extension reaction mixture from its tube and load it carefully on top of the gel material.

- ✓ Spin the column in a microcentrifuge at 730 x g for 2 min.
- ✓ Discard the column. The sample is in the sample collection tube.
- ✓ Dry the sample in a vacuum centrifuge for 10–15 min, or until dry. Do not over-dry.

❖ **Electrophoresis on the ABI PRISM 377 DNA Sequencer:**

- ✓ Prepare 1 L of 10 X TBE pH 8.3 running buffer (Filter using a 1.2 micron filter and de-gas).
- ✓ Prepare a 40 % acrylamide stock (Store at 4°C for 1 month; 38 g of acrylamide and 2 g of bis-acrylamide in 90 mL de-ionized water. Adjust volume to 100 mL. Filter using a 1.2 micron filter and de-gas).
- ✓ Prepare a 5 % sequencing gel (20 x 48 cm) by adding the following components to a 200 mL beaker:

Components	**Amount for one gel**
Distilled water	Make up final volume to 50 mL
Urea	25 g
10 X TBE buffer pH 8.3	5 mL
40 % Acrylamide mix	6.25 mL

Mix components. Filter using a 1.2 micron filter and de-gas. Just before pouring add:

10 % Ammonium persulfate	250 µL
TEMED	34 µL

- ✓ Fill the sequencing chamber using 1X TBE running buffer.
- ✓ Pre-run gel at 2300 V until gel temperature reaches 47°C.
- ✓ Prepare a loading buffer by combining the following in a 5:1 ratio: de-ionized formamide and 25 mM EDTA (pH 8.0) with blue dextran (50 mg/mL).
- ✓ Resuspend each sample pellet (plasmid PCR product) in 6 µL loading buffer if using 64 well combs.
- ✓ Vortex and spin the samples.
- ✓ Heat the samples at 95°C for 2 min to denature. Place on ice until ready to load.
- ✓ Pause the gel run and flush wells with running gel.

- ✓ Load 1 µL of each sample into a separate lane of the gel. Load odd numbered lanes. Run gel for 3 min. Flush wells with running buffer. Load even lanes.
- ✓ Run gel at 2300 V for 10 h at a constant temperature of 51°C.

Appendix 7.10 Oligonucleotide Primer Synthesis Protocol:

The emergence of quick, convenient methods for the synthesis of moderately long oligonucleotides with defined sequences has followed close upon the development of rapid sequencing methods. Chemical synthesis is based on the ability to protect specifically (i.e., to prevent having a chemical reaction occur at) either the 5' or the 3' end of a mono- or oligonucleotide. This is done by hanging a large blocking group onto either the 5' or the 3' hydroxyl. Different blocking groups are used: some can be removed with acid, some with base. Thus, a 5' blocked mononucleotide can be chemically condensed with a 3'-blocked molecule, resulting in a dinucleotide that is blocked at both ends. Either the 5' or the 3' blocking group is then removed (using either acid or base), and the dinucleotide is reacted with an appropriately unblocked mono- or dinucleotide. This cycle of condensation, removal of one or the other blocking group, and recondensation can be repeated many times until an oligonucleotide of the desired length is obtained. Until a few years ago, synthesis of oligonucleotides was a time consuming process that was limited to linking together fewer than 20 nucleotides. Now DNA synthesis is performed using programmable machines that are capable of synthesizing oligoninleotides as long as 100 bases in ~ 10 h. The limiting factors are the progressively lower yields of oligonucleotides with increasing length and the need to purify the products using high performance liquid chromatography (HPLC). The availability of oligonucleotides for use as probes or primers has made possible a wide variety of analytical techniques.

The Beckman Oligo 1000 DNA synthesizer was used to synthesis PCR primers using the basic protocol outlined below. Synthesis of an oligonucleotide by solid-phase phosphoramidite chemistry is outlined in Fig. 7.4. The 3' nucleotide is attached to an inert support of small glass beads, which are placed in a reaction vessel. The oligonucleotide is built up one nucleotide at a time from 3' to 5' by a three step cycle. In the first step, the nucleotide precursor containing base 2 is added to the reaction vessel. The 5' hydroxyl of base 1 couples to the 3' phosphorous of base 2. In the second step, the unstable trivalent phosphite is oxidized to the stable phosphate. In the third step, the dimethoxytrityl group that protects the 5' hydroxyl of the newly added nucleotide is removed, thereby completing one cycle. The process is repeated by addition of the next nucleotide precursor. Finally, the completed oligonucleotide is cleaved from the glass support, and groups protecting the phosphates and bases are removed. Completed oligonucleotides products are purified from reaction components and incomplete products using HPLC. Re-hydrate the vacuum dried primer pellet (final concentration of 5 pmoles/μL) using TE buffer pH 8.0. Prepare 50 μL aliquots of diluted primer in 0.5 mL vials and store at -20°C. Generally, 100 μL PCR reactions require 20 pmoles of each primer (0.2 pmoles/μL).

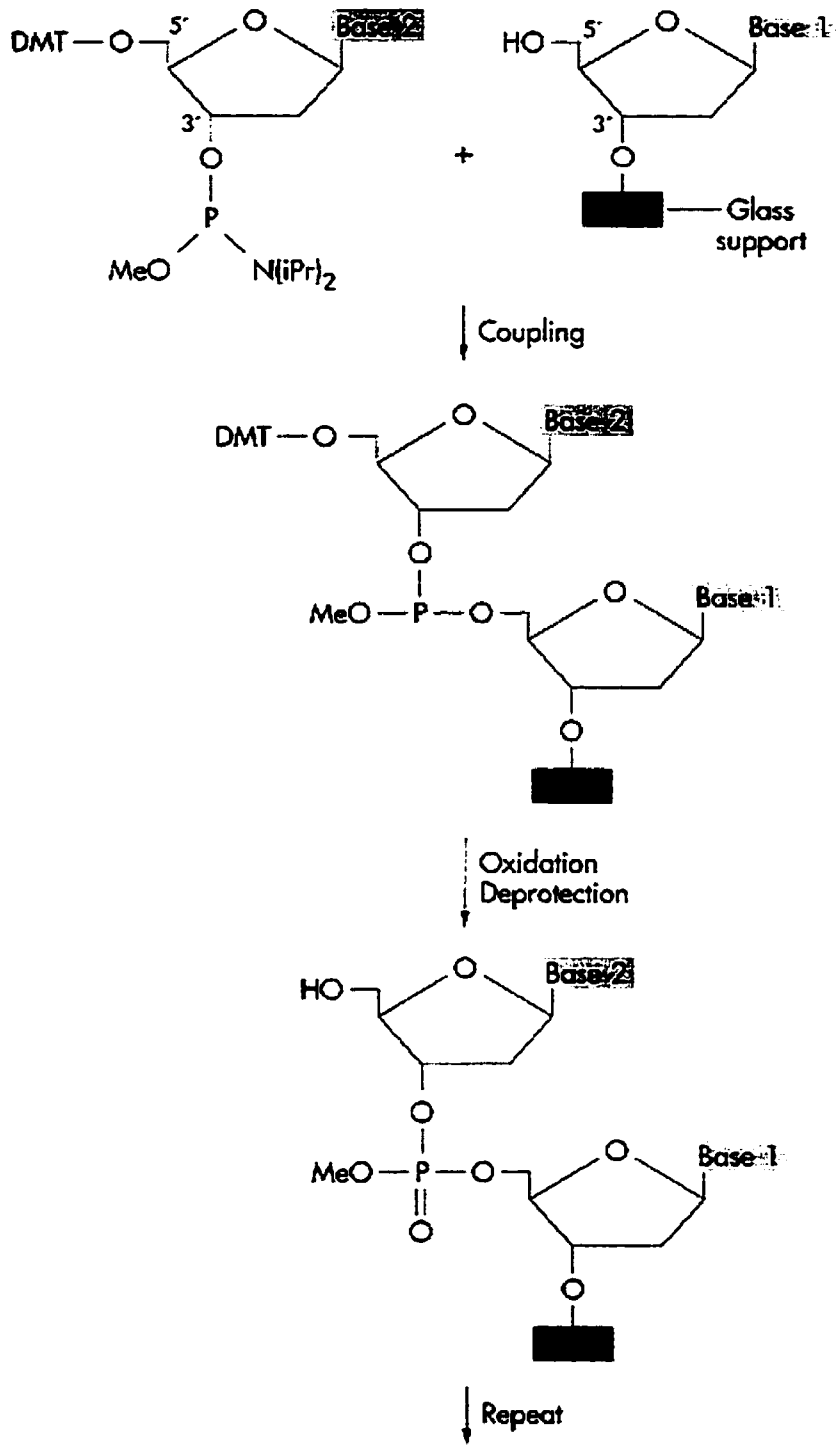


Fig. 7.4. Synthesis of an oligonucleotide by solid-phase phosphoramidite chemistry (Adapted from Watson *et al.*, 1992).

Appendix 7.11 MEGALIGN Program of DNASTAR:

MEGALIGN creates alignments between two or more DNA and/or protein sequences. MEGALIGN reconstructs phylogeny and allows the user to tailor the alignment output for analysis and publication. Alignments are useful in comparing homology, structure, and function between different sequences. If two or more sequences are highly similar, it is likely that they also share common ancestry, morphology, and purpose. For example, all known histone proteins share about 95 % of their residues. From crystallographic and catalytic studies of a few species, a common function and nearly structure has been determined. If the primary sequence of a novel protein is nearly identical to any in this group, then it is fair to say it may share structure, function, and ancestry with characterized histone proteins.

To create a MEGALIGN project, enter sequences in the worktable. Perform a multiple alignment project. Each alignment algorithm functions differently, so keep this in mind when assigning parameters and weights in the alignment. Perform the alignment once the algorithm and its parameters have been selected. After MEGALIGN completes the alignment, the user can begin analysis with an alignment display, phylogenetic tree, and tabular summaries, such as percent divergence, percent similarity and residue substitutions.

MEGALIGN can align multiple sequences using the cluster method algorithm. The cluster algorithm makes no prior assumptions of relatedness. It groups sequences into clusters by examining the distances between all pairs. The clusters are aligned pairwise, then as groups. Use the cluster method to align sequences whose similarity might not necessarily be evolutionary. In the following explanation, pairwise parameters refer to parameters used in scoring alignment pairs in the original matrix. Multiple alignment parameters refer to values used when scoring groups of sequences.

The pairwise alignment parameters have the following options: k-tuple, gap penalty, window, and diagonals saved. A k-tuple is the number of residues compared to each other at a time. A k-tuple of 1 provides a more exhaustive search but takes longer. A higher k-tuple value finds fewer but longer matching regions. A lower k-tuple value finds shorter matching regions. A gap penalty deducts for gaps in the initial pairwise comparisons of the cluster alignment. Use a higher gap penalty to suppress pairwise gapping and a lower gap penalty to encourage gapping. Once a gap is inserted, it can be removed by editing. This forces later clustering to proceed with gaps intact. The window determines over how long a range the program searches for all possible or alternate k-tuples. The search first finds a dominant region of similarity. It uses this region as its starting point and searches out diagonally until it reaches the end of the window. Diagonals are saved. In the pairwise calculation, the diagonals saved are the best scoring diagonals that are

saved for each pair in the matrix. These diagonals are used in the clustering and scoring of ancestors. Saving more diagonals provides more options for MEGALIGN to consider. The multiple alignment parameters have two options: gap penalty and gap length penalty. For each gap introduced in the alignment, the program deducts a penalty from the score. A higher gap penalty suppresses gapping; a lower value promotes it. The program also assesses penalties based on the length of the gap. The more residues the gap spans, the greater the penalty. The program deducts these penalties from the overall score of the alignment. As with the gap penalty, the user can suppress or promote gapping by changing the default value.

In the grouping stages, each method calculates ancestral taxa based on a weight table. Weight tables assign values for comparing residues based on evolutionary substitutions patterns, charge, structural, and chemical similarity. The user needs to select the PAM 250 weight table, the default, prior to performing an alignment. PAM stands for percent accepted mutations. One PAM represents one mutation per 100 residues; therefore, PAM250 means 2.5 mutations per residue. PAM tables are determined empirically by assessing evolutionary changes to sequences known to be closely related.

Once the user have aligned a series of sequences and performed any manual alterations, they can visualize results with MEGALIGN. Two statistical and graphical reports are available: alignment report and sequence distances. The alignment report displays the gaps, sequence names, and the aligned sequences in the project. The sequence distances show a plot of calculated similarity between each sequence pair. The table represents the initial pairwise matrix used in the first step of the multiple alignment. The similarity index compares sequences i and j directly, without accounting for phylogenetic relationships.

Similarity (i, j) = 100 x sum of the matches/length - gap residues (i)-gap residues (j)

Appendix 7.12 Quick Change Site-Directed Mutagenesis Kit:

The Quick change site directed mutagenesis kit can be used to make point mutations and is performed using Pfu DNA polymerase, which replicates both plasmid strands with high fidelity. The kit allows site specific mutation in virtually any double stranded plasmid. This rapid, four-step procedure generates mutants with greater than 80 % efficiency. The protocol is simple to perform and uses mini-prep plasmid DNA. The basic procedure utilizes a supercoiled, double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, extend during temperature cycling by means of Pfu DNA polymerase. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with Dpn I. The Dpn I endonuclease (target sequence 5'-G^{m6}ATC-3') is specific for methylated and hemi-methylated synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to Dnp I digestion. The nicked vector DNA incorporating the desired mutations is then transformed into *E. coli*. The small amount of starting DNA template required to perform this method, the high fidelity of the Pfu DNA polymerase, and the low cycle number all contribute to the high mutation efficiency and decreased potential for random mutations during the reaction. The protocol was performed as follows:

- DNA plasmid preparation of clone GBSSIMMI (2.2 kb insert within 3 kb pBluescript = 5.2 kb) was conducted using the CTAB plasmid isolation method described in appendix 7.4.
- Overlay each reaction with two drops of mineral oil.
- Add the following components to a sterile microfuge tube. Place the tube on ice during pipetting.

Components †	**Amount per reaction**
Distilled water	19.7 µL
Cloned Pfu DNA polymerase 10X reaction buffer ‡	1.8 µL
dNTP mix (10 mM each)	0.5 µL
WxMUTF1 Primer (5 pmoles/µL)	1 µL
WxMUTR1 Primer (5 pmoles/µL)	1 µL
Clone GBSSIMMI (50 pg/µL)	1 µL
Cloned Pfu DNA polymerase (2.5 U/µL) §	1 µL
Total reaction volume	25 µL

† All components stored at -20°C.

‡ 10 X reaction buffer: 0.2 M Tris-HCL (pH 8.8), 0.02 M $MgSO_4$, 0.1 M KCL, 0.1 M $(NH_4)_2SO_4$, 1 % Triton X-100, and 1 mg/mL nuclease-free BSA.

§ Stratagene; One unit of activity is the amount of cloned Pfu DNA polymerase required to incorporate 10 nM of [^{3}H]TTP into an acid insoluble form in 30 min at 72°C. Cloned Pfu DNA polymerase is isolated from the hyperthermophilic marine archaebacterium, *Pyrococcus furiosus*. The multi-functional thermostable enzyme possesses both 5'- to 3'-DNA polymerase and 3'- to 5'-exonuclease activity which results in a 12-fold increase in fidelity of DNA synthesis over Taq DNA polymersae. Cloned Pfu DNA polymerase has a temperature optimum between 72 and 78°C and remains > 95 % active following a 1 h incubation at 95°C.

- Denature the plasmid and anneal the oligonucleotide primers containing the desired mutation. Amplification conditions included 5 min at 95°C, 18 cycles of 30 sec at 95°C, 1 min at 55°C, and 11 min (5.5 kb DNA template x 2 min/kb extension time required by polymerase) at 68°C. Using the non-strand-displacing action of Pfu DNA polymerase, extend and incorporate the mutagenic primers resulting in nicked circular strands. Store at 4°C overnight.
- Digest the methylated, non-mutated parental double-stranded DNA template with Dpn I. Add 1 µL of the Dpn I restriction enzyme (10 U/µL) below the mineral oil overlay. Gently and thoroughly mix each reaction, spin down for 1 min and incubate at 37°C for 1 h. Enzymatic reactions were terminated by heat inactivation at 85°C for 30 min.
- Transform the circular, nicked double stranded DNA into INVαF' *E. coli* cells as described in appendix 7.7. After transformation, the *E. coli* cells repair the nicks in the mutated plasmid.
- Pick 10 colonies for plasmid isolation and restriction analysis.
- Grow colonies overnight in 2 mL LB broth containing 50 µg/mL of ampicillin.
- Isolate plasmid using the CTAB method and confirm presence and incorporation of a second Eco RI restriction site by digesting the isolated plasmid DNA samples with Eco RI restriction endonuclease. Analyze the digestion products on a 2 % agarose gel. A 350 bp DNA fragment will indicate the successful incorporation of the new Eco RI site.

Appendix 7.13 Preparation of Ultra-Competant *E. coli* Cells:

This procedure is based on Inoue *et al.* (1990) and was performed as follows to make competent *E. coli* strains to be used for transformations:

❖ **Preparation of Ultra-Competant *E. coli* Cells**

- ✓ Inoculate a 2 mL LB (100 µg/mL ampicillin) with an *E. coli* strain. Incubate overnight at 37°C with shaking at 225 rpm.
- ✓ Inoculate 250 mL of SOB medium (Dissolve 20 g bacto-tryptone, 5 g yeast extract, 0.58 g NaCl, 0.19 g KCl, 2.0 g $MgCl_2$, and 2.5 g $MgSO_4$ in 950 mL of de-ionized water. Adjust pH to 6.7-7.0. Adjust final volume to 1L. Autoclave.) using an aliquot of the overnight culture. Incubate at 18°C with constant shaking at 225 rpm until $OD_{600} = 0.6$.
- ✓ Place cells on ice for 10 min.
- ✓ Centrifuge at 2500 x g for 10 min at 4°C.
- ✓ Gently resuspend cells in 80 mL of ice cold transformation buffer (Dissolve 3.0 g Pipes, 2.2 g $CaCl_2$, and 18.64 g KCl in 950 mL of de-ionized water. Adjust pH to 6.7 using KOH. Add 10.9 g $MnCl_2$. Adjust final volume to 1 L. Autoclave).
- ✓ Place cells of ice for 10 min.
- ✓ Centrifuge at 2500 x g for 10 min at 4°C.
- ✓ Gently resuspend cells in 20 mL of ice cold transformation buffer.
- ✓ Add dimethyl sulphoxide (DMSO) to a final concentration of 7 %.
- ✓ Place cells on ice for 10 min.
- ✓ Aliquot by pouring cells into 1-2 mL microfuge tubes and freeze in liquid nitrogen.
- ✓ Store at -70°C until needed.

❖ **Transforming Competent *E. coli* Cells with Plasmid DNA:**

- ✓ Remove a vial of competent *E. coli* cells from -80°C storage. Allow cells to completely thaw on ice. Resuspend cells by gently inverting tube.
- ✓ Label 15 mL polypropylene tubes and chill them on ice. Using a chilled pipette tip transfer 100 µL of competent cells into each tube. Controls that were included in each transformation were: (1) competent bacteria that received 2 ng of supercoiled plasmid DNA and (2) competent bacteria that received no plasmid DNA.
- ✓ Add 5-10 µL of ligation reaction to the competent cells. DO NOT PIPETTE MIXTURE. Use the tip of pipette to stir the mixture. Incubate cells on ice for 30 min.

- ✓ Transfer tubes to a rack placed in a 42°C water bath. Heat shock cells for 90 sec. Circulate the water in the bath gently. DO NOT SHAKE TUBES.
- ✓ Rapidly transfer tubes to an ice bath. Allow cells to chill 1-2 min.
- ✓ Add 800 µL SOC medium (Dissolve 20 g bacto-tryptone, 5 g yeast extract, 0.58 g NaCl, 0.19 g KCl, 2.0 g $MgCl_2$,, and 2.5 g Mg SO_4 in 950 mL of de-ionized water. Adjust pH to 6.7-7.0. Adjust final volume to 1L. Autoclave. Add 20 mL of sterile 1M glucose solution. To make the glucose stock solution dissolve 18 g glucose in 90 mL de-ionized water. Adjust volume to 100 mL with de-ionized water. Sterilize by filtration through a 0.22 micron filter.) to each tube. Transfer cultures to a 37°C incubator for 1 h with constant shaking at 225 rpm.
- ✓ Plate up to 200 µL of cells per 90 mm LB plate containing an antibiotic (100 µg/mL). Spread cells on plate.
- ✓ Leave plates at room temperature till liquid has been absorbed.
- ✓ Invert plates and incubate at 37°C. Colonies should appear in 12-16 h. Transfer plates to 4°C for proper blue/white colony color development.
- ✓ Proceed with CTAB plasmid isolation protocol as described in appendix 7.4.

Appendix 7.14 Bio-Rad Protein Assay Protocol:

The Bio-Rad Protein Assay, based on the method of Bradford (measures total protein based on a colorimetric reaction between Coomassie Brilliant Blue G-250 and the protein in the sample), is a simple and accurate procedure for determining concentration of solubilized protein. It involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a spectrophotometer. Comparison to a standard curve provides a relative measurement of protein concentration. The Bio-Rad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine. From 1 to 140 µg/mL of protein can be detected. The standard procedure was performed as follows:

- Prepare 10 mL of a Bovine Serum Albumin (BSA) to a final concentration of 10 mg/mL. Dispense into 300 µL aliquots and store at -20°C until needed.
- Prepare the Bio-Rad Protein Assay Dye Reagent Concentrate (Cat. no. 500-0006) by diluting 1 part dye reagent with 4 parts distilled, de-ionized (DDI) water. Filter through Whatman #1 filter to remove particulates. Dilute 20 mL of Bradford reagent in 80 mL of water.
- Prepare ten 13 x 100 mm test tubes on a test tube rack. Label tubes as 0, 10, 20, 30, and 60. Each tube was done in duplicate.
- Prepare the six BSA protein standards as follows:

Tube no.	BSA (1 µg/µL)	Extraction Buffer †	Water	Total volume
0	0 µL	15 µL	135 µL	150 µL
10	10 µL	15 µL	125 µL	150 µL
20	20 µL	15 µL	115 µL	150 µL
30	30 µL	15 µL	105 µL	150 µL
60	60 µL	15 µL	75 µL	150 µL

† Extraction buffer: buffer in which protein sample of interest is suspended. Interferences may be caused by chemical-protein and/or chemical-dye interactions between chemicals in the extraction buffer and protein in the standards or sample. Thus, it is important to test the affect of each new extraction buffer on the protein assay reaction.

- Prepare six 13 x 100 mm test tubes on a test tube rack per protein sample of interest. Label tubes as 5, 10, and 15. Each tube was done in duplicate.
- Prepare the six test reactions of each protein sample as follows:

Tube no.	Protein Sample †	Water	Total volume
5	5 µL	145 µL	150 µL
10	10 µL	140 µL	150 µL
15	15 µL	135 µL	150 µL

† Resuspeneded in the same extraction buffer that was used to set up the six protein standards.

- Add 5 mL of the diluted dye reagent to each standard and protein sample tube. Vortex each tube and incubate 5 min at room temperature. Absorbance will increase over time; samples should not be incubated at 24°C for more than 1 h.
- Measure the absorbance at 595 nm of the protein standards using 3.5 mL disposable cuvettes (Cat. no. 223-9950). Use the duplicate protein standard containing no BSA (0 µg) to blank the DU Series 7400 Beckman Spectrophotometer at 260 nm. The protein analysis mode calculates a linear standard curve, as defined

$$\text{Absorbance}_{595\ nm} = (\text{Concentration} \times \text{slope}) + \text{A-intercept}$$

where concentration is the concentration of a known protein standard, slope is the slope of the calibration line, and A-intercept is the absorbance value that is intercepted by the calibration line.

- BSA standards were used to set up a linear relationship between absorbance at 595 nm and protein concentration (µg/µL). The linear range of the assay for BSA has been set up to be 10-60 µg.
- Measure the absorbance at 595 nm of the protein samples of interest using 3.5 mL disposable cuvettes. Protein concentrations (µg/µL) were extrapolated from the absorbance vs. protein concentration calibration curve. Each protein concentration value needs to be divided by the volume of protein sample (i.e., 5, 10, or 15 µL) used to perform the assay. The final protein concentration represents the amount of protein in the sample in µg/µL.
- Immediately use protein samples to conduct enzyme assays.

- Protein samples can be aliquoted (30 µg/vial) and stored at - 20°C if re-suspended in 10 % glycerol. These frozen samples can be used for SDS-PAGE analysis.

Appendix 7.15 Starch Granule Isolation:

Starch granule isolation was performed as described by Zhao and Sharp (1996) as described below:

- In a 1.5 m L microfuge tube, soak two to three mature grains in 1 mL distilled water at 4°C overnight.
- Centrifuge at 15 000 x g for 5 min and discard supernatant.
- Crush pellet using a small plastic pestle. Re-suspend in 500 µL distilled water.
- Layer on 1 mL of 80 % cesium chloride solution and centrifuge at 15000 x g for 5 min to pellet starch. Discard supernatant. Re-suspend starch pellet in 300 µL of water. Repeat cesium chloride purification two more times.
- Remove the supernatant and wash the starch pellet with 1 mL washing buffer I (0.055 M Tris-HCL pH 6.8, 2.3 % SDS, and 10 % Glycerol; Add 5 % ß-mercaptoethanol just prior to use of buffer). Centrifuge at 15000 x g for 5 min. Repeat wash once again.
- Wash three times with distilled water, once with acetone, and vacuum dry 10 min. Centrifuge at 15 000 x g for 5 min after each wash. Store with a desiccant at 4°C.

Appendix 7.16 One-Dimensional SDS Polyacrylamide Gel Electrophoresis:

Gel electrophoresis is one of the most frequently used and most powerful techniques in laboratory research. This method separates biomolecules in complex mixtures according to their physical properties of size and charge. It is helpful to have a basic understanding of electrophoresis concepts so that when a new sample is being assessed, a logical approach can be taken in selecting the proper tools. This section provides some basic theoretical aspects of gel electrophoresis.

During electrophoresis, there is an intricate interaction of sample, gel matrix, buffers, and electricity resulting in separate bands of individual molecules. Applications for electrophoresis are very broad, including protein, nucleic acid, and carbohydrate work. Protein electrophoresis is generally performed in polyacrylamide gels, while nucleic acid electrophoresis generally uses agarose gels, although TBE polyacrylamide gels are common for resolving DNA fragments of 50-2000 bp sizes. Polyacrylamide gels are composed of long linear polyacrylamide chains crosslinked with bis-acrylamide to create a network of pores interspersed between bundles of polymer. The structural features of a gel can be thought of as a three-dimensional sieve, made up of random distributions of solid material and pores. The ability of proteins or nucleic acids to move through the gel depends on their size and structure, relative to the pores of the gel. Large molecules can usually be expected to migrate more slowly than small ones, creating separation of the distinct particles within the gel.

By convention, polyacrylamide gels are characterized by % T, which is the weight percentage of total monomer including crosslinker (in g/100 mL). The % T gives an indication of the relative pore size of the gel. In general, pore size decreases with increasing % T. The % T can be calculated by the following equation:

% T = grams of acrylamide + grams of bis-acrylamide/total volume (mL) x 100

The polyacrylamide % T can be made as a single continuous percentage throughout the gel from 7.5 up to 20 %. % C is the crosslinker ratio of the monomer solution. The % C can be calculated by the following equation:

% C = grams of bis-acrylamide/grams of acrylamide + grams of bis-acrylamide x 100

In general, pore size decreases with increasing % C. The choice of proper acrylamide concentration is critical to the success of the separation. Too high % T can lead to exclusion of proteins from the gel, and too low % T can decrease sieving.

The electrolyte buffer is a very important part of the electrophoresis system. It determines power requirements and affects separation. Sample proteins vary widely in their response to the constituents and make-up of the buffer. The buffer system is composed of both the buffer used in the gel and the running buffer. Discontinuous buffer systems were devised initially for use with undenatured, or native, proteins. By using different buffers in the gel and in the electrode solutions, and adding a stacking gel to the resolving gel, the samples can be compressed into a thin starting band, from which finely resolved final bands of individual proteins separate. A discontinuous gel has two main parts. The upper gel is known as a stacking gel and is made of a large pore matrix (typically 5 % T) that acts as an anti-convective medium. The proteins pass easily through this matrix between leading and trailing ion fronts. Proteins become much more compressed into narrow starting zones by this method than is possible with any mechanical means. The lower resolving gel is made of a dense matrix that acts as a sieve. To overcome the net charge effect (where high molecular weight proteins with high net negative charge migrate further into the gel than lower molecular weight, less strongly charged, proteins) on protein gels sodium dodecyl sulfate (SDS), a detergent, is incorporated into the system. SDS is a strong protein denaturing detergent. SDS is often used in biochemical preparations because it binds tenaciously to proteins (about 1.4 g SDS/g of protein or about one SDS molecule for every two amino acid residues) causing them to assume a rod-like shape. The large negative charge that the SDS imparts masks the protein's intrinsic charge so that SDS-treated proteins tend to have identical charge-to-mass ratios and similar shapes. In the denaturing (SDS) discontinuous system, proteins are denatured by heating them in buffer containing SDS and thiol reducing ß-mercaptoethanol (reductively cleave disulfide bonds, S-S, formed between Cys residues of a polypeptide or more than one polypeptide). The resultant polypeptides take on a uniform charge-to-mass ratio and rod-like dimensions imparted by the SDS, proportional to their molecular weights.

The denaturing (SDS) discontinuous system can be utilized to estimate the molecular weight of a protein of interest. Molecular weights are determined in SDS-PAGE by comparing the mobilities of test proteins to the mobilities of known protein markers. The relative mobility, R_f, is defined as the mobility of a protein divided by the mobility of the ion front. Because the ion front is difficult to locate in practice, mobilities can be normalized to the tracking dye that migrates only slightly behind the ion front:

$$R_f = \text{distance to band/distance to dye front}$$

In each gel, a lane of standard proteins of known molecular weights is run in parallel with the test proteins. Plots of the logarithm of protein molecular weight (log Mr) versus the relative mobility, R_f, fit reasonably straight lines. The R_fs of the test proteins are then interpolated into the standard curve to give their approximate molecular weights.

Regulated DC power supplies for electrophoresis should control voltage, current, and power. All modes of operation can produce satisfactory results, but for best results and good reproducibility, some form of electrical control is important. The choice of which electrical parameter to control is almost a matter of preference. The major limitation is the ability of the cell to dissipate the heat generated by the electrical current during an electrophoretic run. This Joule heat can have many deleterious consequences, such as band distortion, increased diffusion, enzyme inactivation, and protein denaturation. In general, electrophoresis should be performed at voltage or current settings at which the run proceeds as rapidly as the chamber's ability to draw off heat allows. Constant current conditions, as a rule, result in shorter but hotter runs than do constant voltage runs. The increased run times of constant voltage conditions give increased time for the proteins to diffuse, but this appears to be offset by the temperature-dependent increase in diffusion rate of the constant current mode.

A general overview of the stages in protein separation using denaturing discontinuous gels is described as follows. (1) Denatured sample proteins are loaded into the wells. (2) Voltage is applied and the samples move into the gel. The chloride ions already present in the gel run faster than the SDS-proteins and form an ion front. The glycinate ions flow in from the running buffer and form a front behind the proteins. (3) A voltage gradient is created between chloride and glycinate ions, which sandwich the proteins between. (4) The proteins are tightly stacked between the chloride and glycinate ion fronts. At the interface between the stacking and resolving gels, the percentage of acrylamide increases and the pore size decreases. Movement of the proteins into the resolving gel is met with increased resistance. (5) The smaller pore size resolving gel begins to separate the proteins based on molecular weight only, since the charge-to-mass ratio is equal in all the protein sample. (6) The individual proteins are separated into band patterns ordered according to molecular weight.

One-dimensional SDS Polyacrylamide Gel Electrophoresis was performed as described by Zhao and Sharp (1996) as described below:

- Wash two Protean II xi Bio-Rad outer (18.3 x 20 cm) and two inner (16 x 20 cm) plates with soap, rinse well, and spray with 70% ethanol. Allow to air dry.
- Place 0.75 mm spacers between gel plates and clamp in place using apparatus setter.

- Prepare 45 mL of a 16 % (% T = 16) or 20 % (% T = 20) resolving gel (0.75 mm) solution by adding the following components to a 50 mL Falcon tube:

Components	Amount for two 16 % gels	Amount for two 20 % gels
Distilled water	8.8 mL	2.8 mL
1.5M Tris-HCL, pH 7.8	11.3 mL	11.3 mL
10% SDS	450 µL	450 µL
30 % Acrylamide:		
0.1 % Bis-acrylamide	24 mL	30 mL
Mix components and just before pouring add:		
10 % Ammonium persulfate	450 µL	450 µL
TEMED	18 µL	18 µL

- Pour resolving gel mixture using a 10 mL pipette till the mixture reaches ~ 5 cm from the top of the gel.
- Add drops of iso-butanol onto the resolving gel layer. Allow gel to set 30 min.
- Pour off the overlay and wash six times with de-ionized water. Remove any remaining water with the edge of a piece of filter paper.
- Prepare 24 mL of a 5 % stacking gel (0.75 mm) solution by adding the following components to a 50 mL Falcon tube:

Components	Amount for two 5 % gels
Distilled water	16.2 mL
1 M Tris-HCL, pH 6.8	3 mL
10 % SDS	240 µL
29 % Acrylamide:1 % Bis-acrylamide	4.2 mL
Mix components and just before pouring add:	
10 % Ammonium persulfate	240 µL
TEMED	24 µL

- ❖ Wash 14 well (0.75 mm thick, 18 mm long , 8 mm wide) combs with water and dry with ethanol. Pour stacking gel mixture onto the polymerized resolving gel. Remove air bubbles and insert the comb. Let polymerize for 30 min.
- ❖ If gel cannot be run the same day then wrap gels, without removing the combs, in saran wrap and store at 4°C until next day.
- ❖ Prepare 4 L of Tris-glycine running buffer (25 mM Tris base pH 8.0, 533 mM Glycine pH 8.3, and 0.1 % SDS). Stir at 4°C until needed.
- ❖ While the stacking gel is polymerizing prepare the protein samples for loading onto gel as follows:
  - ✓ For gels to be Silver stained, prepare the samples by heating 10 mg of starch to 100°C for 10 min in 140 µL extraction buffer (0.625M Tris-HCL pH 6.8, 4 % SDS, 10 % glycerol, and 0.005 % bromophenol blue; add 5 % ß-mercaptoethanol just prior to use of buffer) to denature the proteins. (Concurrently prepare and denature a sample containing marker proteins of known molecular weight). Cool the gelatinized starch samples on ice for 5 min and centrifuge at 15 000 x g for 20 min at 4°C. Load 17 µL of supernatant/lane (avoid loading the gelatinized starch pellet).
  - ✓ For gels to be Coomassie stained, prepare 10 mg of starch in 280 µL extraction buffer and load 70 µL of supernatant/lane. Other steps are the same as described above.
  - ✓ For gels to be Western blotted, prepare 10 mg of starch in 140 µL extraction buffer and load 10 µL of supernatant/lane. Other steps are the same as described above.
  - ✓ A sample containing a final concentration of 1 µg (silver staining) or 5 µg (Coomassie staining) of Bio-Rad High range protein standard (Cat. no. 161-0303; standard includes five proteins with molecular weights of 200, 116, 97, 66, and 45 kD) was prepared in 15 µL of extraction buffer and denatured at 95°C for 10 min. A Bio-Rad High range pre-stained protein standard was loaded onto gels to be used for Western blotting in order to determine the orientation of the gel and to verify transfer efficiency.
- ❖ Remove the combs. Wash wells with distilled water to remove unpolymerized acrylamide. Straighten the teeth of the stacking gel. Mark wells with marker. Mount gel into the electrophoresis apparatus.
- ❖ Test for leaking by adding distilled water to the top of the running apparatus.
- ❖ Put the gels in the running chamber. Add running buffer to the top and bottom reservoirs. Disperse air bubbles from the bottom of gel plates.
- ❖ Load samples into the bottom of the wells.

- Connect cooling bath hoses to Protean II xi Bio-Rad vertical electrophoresis apparatus. Turn on cooling chamber. Wait till the gel apparatus fills with water and fill cooling chamber to brim with distilled water. Set temperature at 10°C. Set the Bio-Rad 3000 xi power supply at a constant current of 15 mA/gel or 30 mA/2 gels for 21 h.
- Disassemble gel sandwich by prying the plates apart. Remove stacking gel after making transfer buffer for Western blot and/or fixative for silver stain or Coomassie Brilliant Blue stain.

Appendix 7.17 Protein Detection in Coomassie Staining:

Polypeptides separated by SDS-polyacrylamide gels can be simultaneously fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue R250 dye. The gel is immersed for several hours in a concentrated methanol/acetic acid solution of the dye, and excess dye is then allowed to diffuse from the gel during a prolonged period of destaining. Staining was performed as described by Sambrook *et al.*(1982) as follows:

- Dissolve 0.25 g of Coomassie Brilliant Blue R250 (Fisher Scientific) in 90 mL of methanol:water (1:1 v/v) and 10 mL of glacial acetic acid.
- Immerse the gel in at least 5 volumes of staining solution and place on a slowly rotating platform overnight at room temperature.
- Remove the stain and save it for future use. Destain the gel by soaking it in the methanol/acetic acid solution described above without the dye on a slowly rocking platform for 4-8 h, changing the destaining solution three or four times.
- The more thoroughly the gel is destained, the smaller the amount of protein that can be detected by staining with Coomassie Brilliant Blue. Destaining for 24 h usually allows as little as 0.1 μg of protein to be detected in a single band.
- After destaining, gels may be stored indefinitely in a 20 % glycerol solution in a sealed plastic bag without any diminution in the intensity of staining. Stained gels should not be stored in destaining buffer, which will cause the stained protein bands to fade.
- Photograph the stained gel to make a permanent record.

Appendix 7.18 Protein Detection in Western Blotting:

When proteins are transferred from a gel onto a membrane, they are readily accessible to antibody probes. This has led to the development of a variety of highly specific and sensitive assays collectively known as blots. Probing of membrane-bound proteins is generally done immunologically with antibodies, and is known as immunoblotting or Western blotting. A typical immunoblotting experiment consists of six interrelated steps. (1) Proteins are first fractionated by electrophoresis in a polyacrylamide gel. (2) The proteins are then transferred from the gel to a membrane where they become immobilized as a replica of the gel's band pattern. (3) Next, unoccupied protein-binding sites on the membrane are saturated to prevent non-specific binding of antibodies. (4) The blot is then probed for the proteins of interest with specific, primary antibodies. (5) Secondary antibodies, specific for the primary antibody type and conjugated to detectable reporter groups, such as enzymes, are then used to label the primary antibodies. (6) Finally, the labeled protein bands are made visible by the bound reporter groups acting on an added substrate. As little as 1-5 ng of an average-sized protein can be detected by Western blotting. The following protocol was used for the detection of proteins by Western blotting:

❖ **Transfer of Denatured Proteins to Membrane:**

- ✓ Prepare two 0.75 mm gels, one for Western blotting and the other for silver or Coomassie Brilliant Blue R250 staining, as described in appendices 7.21 and 7.17, respectively.
- ✓ Prepare 3 L of transfer buffer (25 mM Tris base, 192 mM glycine, 0.05 % SDS, and 20 % methanol).
- ✓ Pre-chill transfer buffer, with constant stirring, at 4°C before performing the transfer.
- ✓ Powder must be washed off gloves at all times. Avoid touching gel or membrane with hands and metal objects (e.g., tweezers or scalpel). Instead use a clean glass rod to manipulate the gels.
- ✓ Remove the stacking gel from the gel to be transferred.
- ✓ Wash a plastic container using detergent and rinse well. Equilibrate gel 30 min at 24°C in 200 mL of transfer buffer. Avoid keeping gel in buffer too long since the gel will expand.
- ✓ Pre-wet the Immobilon-P membrane (Millipore; 0.45 micron pore; hydophobicity from the polyvinylidene difluoride [PVDF] in the membrane) in 100 % methanol or an alternative organic solvent for (1-2 sec) until the entire membrane is translucent. The hydophobicity of the PVDF membranes makes it impossible to wet the membrane with

aqueous solutions. Submerge the membrane using ~ 200 mL of transfer buffer for 15 min at room temperature with gentle shaking. At this point the membrane is ready to bind proteins in any blotting application. Do not allow membrane to dry out.

- ✓ Use detergent to wash all parts of the transfer apparatus, gel cassettes, and fiber pads. Rinse with distilled water. This step is used to reduce background on the blot.
- ✓ Wearing gloves, set up the transfer apparatus as follows:
  - ♦ Lay the bottom electrode (which will become the anode, positive, or red coded end) flat on the bench, graphite side up:
  - ♦ Place on the electrode a porous pad, three sheets of 3MM paper that have been soaked in transfer buffer. Stack the sheets one on top of the other so that they are exactly aligned. Using a glass pipette as a roller, squeeze out any air bubbles.
  - ♦ Place the membrane filter on the stack of 3MM paper. Make sure that the filter is exactly aligned and that no air bubbles are trapped between it and the 3MM paper.
  - ♦ Orient the gel so that the mark on the filter corresponds to the bottom left-hand corner of the gel. Squeeze out any air bubbles with a gloved hand.
  - ♦ Place the final three sheets of 3MM paper on the gel, again making sure that they are exactly aligned and that no air bubbles are trapped.
  - ♦ Place a porous pad on the three 3MM paper sheets. Remove air bubbles.
- ✓ Place the upper electrode (which will become the cathode, negative, or black coded end) on top of the stack, graphite side down. Connect the electrical leads (positive or red lead to the anode electrode). Fill the electrophoresis tank with cold transfer buffer.
- ✓ Connect transfer system to a cooling bath set at 4°C.
- ✓ Transfer at 30 V for 6 h with constant cooling at 4°C.
- ✓ Turn off the electric current and disconnect the leads. Disassemble the transfer apparatus from the top downward, peeling off each layer in turn. Transfer the gel to a tray containing Coomassie Brilliant Blue, and stain it as described in appendix 7.17. This will allow the user to check whether electrophoresis transfer is complete.
- ✓ Mark the orientation of the gel by cutting off the top left-hand corner of the membrane. Remove the membrane from the sandwich and transfer it to a clean piece of 3MM paper. Allow the membrane to dry for at least 2 h before proceeding to the detection of the proteins to improve the retention of proteins on the filter during subsequent processing. If the user does not plan to probe the membrane within a day then store the membrane between 2 pieces of filter paper until needed.

Just as proteins transferred from the SDS-polyacrylamide gel can bind to the PVDF filter, so can proteins in the immunological reagents used for probing. The sensitivity of Western blotting depends on reducing this background of non-specific binding by blocking potential binding sites with irrelevant proteins. Of the several blocking solutions that have been devised, the best and least expensive is non-fat dried milk. It is easy to use and is compatible with all of the immunological detection systems in common use. The Western blot will be probed in two stages. An unlabeled antibody specific to the target protein is first incubated with the filter in the presence of blocking solution. The filter is then washed and incubated with a secondary reagent-anti-immunoglobulin that is coupled to an alkaline phosphatase (AP) enzyme. After further washing, the antigen-antibody-antibody complexes on the filter are located by an enzymatic reaction. The substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) is de-phosphorylated into a dense blue compound by immunolocalized alkaline phosphatase. NBT is an electron acceptor in an NBT-BCIP reaction.

❖ **Incubate Filter with Primary Antibody:**

- ✓ Prepare 1 L of blocking buffer A (100 mM Tris Cl, pH 7.5, 225 mM NaCl, 5 % [w/v] non-fat dried milk, and 0.1 % Tween 20).
- ✓ Place the filter in a clean plastic dish which is appropriate for the dimensions of the filter. Incubate the filter for 2 h in 150 mL of blocking buffer A at 24°C with gentle agitation on a platform shaker.
- ✓ Discard the blocking buffer A. Immediately incubate the filter with a primary antibody directed against the target protein (e.g., rabbit antiserum prepared using a 59-60 kD Wx-D1 protein isolated from the endosperm starch of wheat kernels [Demeke *et al.*, 1997a]; Appendix 7.28). Dilute the primary antibody in blocking buffer A (1:2500) by adding 20 µL of antibody to 50 mL of blocking buffer A. Incubate at 24°C with constant agitation for 2 h. Excessive freeze-thawing of the primary antibody, stored in 0.5 mL aliquots at -80°C, can reduce the efficiency of detection of the protein of interest. The background of non-specific binding increases as a function of the time and temperature of incubation.
- ✓ Wash membrane four times for 10-15 min, with constant agitation, using 200 mL blocking buffer A each wash.

❖ **Incubate Filter with Secondary Antibody:**

- ✓ Replace last wash solution with a diluted secondary antibody solution. Dilute secondary antibody (Goat Anti-Rabbit Alkaline Phosphatase-conjugated antibody; Stratagene; Cat

no. 200374) in blocking buffer A (1:5000) by adding 10 µL of antibody to 50 mL of blocking buffer A. Incubate 3 h at 24°C with constant agitation. (Note: The goat antibodies to rabbit IgG are isolated from serum by affinity chromatography using agarose-immobilized antigen. All non-specific antibodies and other serum proteins have been removed. The antibodies have been directed against the whole IgG molecule and react with both heavy and light chains.)

- ✓ Wash membrane four times for 10-15 min, with constant agitation, using 200 mL of blocking buffer A each wash.

❖ **Visualization of the Chromogenic Substrate:**

- ✓ Prepare 200 mL of blocking buffer B (100 mM Tris Cl, pH 7.5, 225 mM NaCl, and 5 % [w/v] non-fat dried milk; Tween 20 interferes with the color development in procedure). Wash membrane twice for 15 min, with constant agitation, using 100 mL blocking buffer B each wash to remove excess Tween 20.
- ✓ Prepare 100 mL of alkaline phosphate buffer (100 mM Tris HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$). Rinse filter with 10 mL of alkaline phosphate buffer three times to remove excess non-fat milk.
- ✓ Prepare chromogenic substrate mixture by diluting NBT (0.7 % final conc.; Stratagene; Cat. no. 300130) and BCIP (0.3 % final conc.; Stratagene; Cat. no. 3000045) in alkaline phosphate buffer. Add 0.1 mL of chromogenic substrate mixture per square inch of filter. Incubate filter in the dark at 24°C with shaking for 10-30 min until the desired band intensity is reached. The chromogenic substrate mixture should be used within 30 min. If the membrane is left in the color development solution too long, excess precipitate formed by the enzyme can settle out on the membrane and cause high background.
- ✓ Immediately rinse the filter with water, dry, and photograph.

Appendix 7.19 Over Expression of the Waxy Protein in RH98 Bacterial Strain:

The RH98 bacterial strain (glycogen starch synthase deficient) originally obtained from Dr. R. Hengge-Aronis (Dept. of Biology, Univ. of Konstanz, Germany; Muffler *et al.* 1997) contained plasmid pGP1-2 which confers kanamycin resistance (Edwards *et al.* 1995):

- ✓ Revive the RH98 cells stored at -80 °C by scraping off solid ice with a sterile wire loop.
- ✓ Streak the splinters on to an LB plate containing kanamycin (10 ug/mL). Incubate at 37 °C overnight.
- ✓ Re-streak cells until large single colonies are growing on the LB plate. Re-streak the cells each week to maintain the viability of the bacterial strain.

Competent RH98 cells (mutant strain), prepared as outlined in appendix 7.13, were transformed with pKK388-1 or pM*Wxl* vectors which confer ampicillin resistance. RH98 containing pKK388-1 was the negative control. Competent JM101 cells (wildtype strain) were transformed with pKK388-1 (positive control). Over expression of the Wx protein in RH98 cells was performed as follows:

- ✓ Inoculate a 45 mL LB culture containing (100 µg/mL ampicillin) in 50 mL Falcon tube using a single colony of RH98 containing pM*Wxl*. Prepare separate tubes for negative and positive controls. Incubate at 37°C for 11 h with shaking at 250 rpm to an OD_{600} = 0.6.
- ✓ Induce cultures with a final conc. of 5 mM IPTG. Incubate at 27°C at 250 rpm 5 h.
- ✓ Collect cells by centrifugation at 12 000 x g for 10 min using 50 mL centrifuge tubes at 4°C.
- ✓ Resuspend cells in 500 µL of ice-cold extraction buffer (9.8 mL 50 mM Tris-acetate pH 8.5 and 10 mM EDTA, 0.5 g Sucrose [5 % w/v], 50 µL 1 M DTT, 57 µL PMSF [100 µg/mL], 20 µL Leupeptin [2 µg/mL]).
- ✓ Lyse cells (3 x 20 sec sonication) and remove cell debris by centrifugation for 15 min 15 000 x g at 4°C.
- ✓ Determine Protein content as described in appendix 7.14.

Appendix 7.20 Starch Synthase Assay:

The transfer of glucose onto exogenous primer (amylopectin) from ADP-glucose was measured in a total reaction volume of 0.2 mL containing: 70 nmol ADP [^{14}C] glucose (Amersham Life Science; Specific activity of 627 cpm/nmol; Adenosine diphospho-D-{U-^{14}C]glucose, ammonium salt is prepared by enzymatic synthesis from alpha-D-[U-^{14}C]glucose-1-phosphate), 0.1M bicine (pH 8.5), 0.025 M potassium acetate, 0.01 M GSH, 0.005 M EDTA, 0.01 mM DTT, 0.001 g potato amylopectin (Sigma Type III), and crude protein extract. Starch synthases (EC 2.4.1.21) catalyze the following generalized reaction:

$$\text{ADP-glucose} + (\text{glucosyl})_n \longrightarrow \text{ADP} + (\text{glycosyl})_{n+1}$$

A generalized enzyme assay has several distinct parts described below. (1) The reaction mixture is prepared and the reaction can be started by the addition of the substrate. (2) The reaction is incubated at a specific temperature for a pre-determined time. (3) The enzymatic reaction is terminated by heat inactivation. (4) The substrate is washed away from the product of the incubated mixture using a methanol:KCL solution. Large polymers (i.e., Glucan product) bind to filter paper and small polymers (i.e., radioactive ADP [^{14}C] glucose substrate) do not bind to the filter paper. Non-incorporated substrate is washed off using a methanol: KCL solution. (5) The measurement of radioactivity in each sample is done using a scintillation counter. (6) The progress of the reaction is given by the amount of radioactivity product recovered.

The following starch synthase enzyme assay is based on Denyer *et al.* (1995):

- Prepare a reaction mixture on ice for the appropriate number of triplicated samples to be assayed. The recipe is for one reaction and must be adjusted for multiple reactions, including the number of triplicated sample reactions, positive controls, and negative controls plus an additional reaction volume. ADP(U-^{14}C) glucose (Fig. 7.5) was added to individual reaction tubes to activate the reactions.

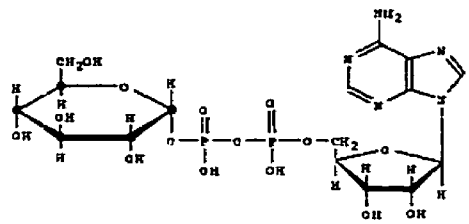


Fig. 7.5 Structure of ADP-D-[U^{14}C] glucose.

Components	Amount per reaction
Distilled Water	110 µL
Potato amylopectin †	0.001 g
0.5 M bicine, pH 8.5	40 µL
0.25 M potassium acetate	20 µL
1 M DTT	2 µL
0.5 M EDTA, pH 8.0	2 µL
Glutathione (GSH)	0.00061 g
Crude protein extract (3, 15, 30, or 60 µg)	25 µL
ADP(U-14 C) Glucose(0.32 µCi/20 µL aliquoted/vial)	1 µL
Total reaction volume	200 µL

† Add the amylopectin and distilled water and heat the solution 30 min at 90°C to solubilize the amylopectin. Occasionally invert tube gently 5 times to mix contents.

- Mix the bulk reaction mixture well. The final volume of each sample reaction is 200 µL. Aliquot 175 µL of the bulk reaction mixture into the appropriate number of sterile 1.5 mL screw cap tubes and place the tubes on ice.
- Dilute 3, 15, 30, or 60 µg of crude protein extract in a total volume of 25 µL of extraction buffer. Add the appropriate protein concentration to the appropriately labeled tube. Cap tubes and gently vortex the tubes to mix the reaction components.
- For each protein concentration, triplicated assay mixtures containing heat-denatured cell extracts (3, 15, 30, or 60 µg) were used as negative controls.
- Add 1 µL of ADP(U-^{14}C) glucose per reaction and incubate the activated reactions in a water bath set at 25°C for 30 min.
- Stop reactions by heating for 5 min at 100°C.
- Aliquots of 100 µL (note: multiple the detected counts per min of each sample by 2 to measure the activity in the total 200 µL reaction) were removed from each reaction and absorbed on Whatman 31 ET filter discs (1 inch sq.), which were washed 4 X 30 min with 75 % Methanol/ 1 % KCL solution.

- Wash 10 filters per 1 L beaker at a time with 800 mL 75 % Methanol 1 % KCl for 30 min. Repeat wash 4 times in total to remove unincorporated ADP[^{14}C] glucose.
- Put control filters in 15 mL of Opti-fluor scintillation liquid (Packard) contained in plastic vials. The starch synthase activity was calculated as nmol of ADP-glucose incorporated into glucan per milligram of protein per min after subtraction of background values obtained from heat denatured extracts.
- Differential incorporated ADP[^{14}C]-glucose into glucan was determined by reading the samples in plastic vials on a 1219 RackBeta Liquid Scintillation Counter (Fisher Scientific). The basic principle behind the counter monitor is that the radioactive decay gives rise to multiple photon reactions as one disintegration releases energy sufficient for many photons to be produced in the scintillation cocktail. Note: It is important that the discs are washed carefully to remove the unincorporated ADP[^{14}C]-glucose because the detector can not differentiate between the radiolabel of the ADP[^{14}C]-glucose (i.e., substrate) and that of the glucan (i.e., product).
- The counts per minute (cpm) data used to calculate the amount of ADP [^{14}C]-glucose incorporated into glucan using four different crude protein concentrations from *E. coli* strains JM101 and RH98 transformed with pKK388-1 or pM*WxI* is presented in Table 7.4. The following equation was used to calculate the nmol (^{14}C) glucose incorporated per mg of protein per minute of incubation:

$$\text{nmol } (^{14}\text{C}) \text{ glucose mg}^{-1} \text{ min}^{-1} = (\text{cpm}_S - \text{cpm}_{NC})/627 \text{ cpm/nmol} \times 1/\text{mg protein} \times 1/\text{min}$$

where cpm_S is the counts per minute of a given sample, cpm_{NC} is the counts per minute of the heat denature negative control, 627 cpm/nmol is the specific activity of the ADP(U-^{14}C) glucose, mg protein is the milligrams of total protein extract used per reaction (0.003, 0.015, 0.03, or 0.06 mg in this study), and min is the time reactions were incubated at 24°C.

- **Statistical Differences Between Means:**

  Compare means using an unpaired t-test as described in appendix 7.22.

Table 7.4. Counts per minute used to calculate starch synthase activity using different protein concentrations from JM101 and RH98 (transformed with pKK388-1 or pM*Wxl*) †.

Sample	Replication	Soluble protein extract (µg/200 uL reaction)			
		3	15	30	60
RH98 pKK388-1	1	280	304	506	518
	2	262	360	370	550
	3	224	338	404	470
	Mean	**256**	**334**	**426**	**512**
RH98 pM*Wxl*	1	246	244	314	310
	2	238	286	318	296
	3	238	252	236	250
	Mean	**240**	**260**	**290**	**286**
Positive control: [JM101 pKK388-1]	1	25 132	45 318	49 512	50 654
	2	27 524	47 712	49 930	50 580
	3	28 020	48 670	50 286	51 460
	Mean	**26 892**	**47 234**	**49 910**	**50 898**
Negative control: [JM101 pKK388-1]	1	256	252	306	304
	2	294	278	262	288
	3	274	256	280	262
	Mean	**274**	**262**	**282**	**284**

† The half-life of ^{14}C is 5760 years.

Appendix 7.21 Protein Detection in Silver Staining:

A number of methods have been developed to stain polypeptides with silver salts after separation by SDS-PAGE. In every case, the process relies on differential reduction of silver ions that are bound to the side chains of amino acids. The following method makes use of silver nitrate. Silver staining is approximately 100- to 1000-fold more sensitive than staining with Coomassie Brilliant Blue R250 and is capable of detecting as little as 0.1-1.0 ng of polypeptide in a single band.

- Wear gloves and handle the gel gently because pressure and fingerprints will produce staining artifacts. In addition, it is essential to use clean glassware and de-ionized water because contaminants can greatly reduce the sensitivity of silver staining.
- Separate the proteins by electrophoresis through an SDS-polyacrylamide gel as described in appendix 7.16. Fix the proteins by incubating the gel for 30 min at 24°C with gentle shaking in a 300 mL of a solution of methanol:glacial acetic acid:water (50:10:40).
- Discard the fixing solution using aspiration, and add 300 mL of a solution of methanol:glacial acetic acid:water (5.5:7.5:87). Incubate the gel 30 min at room temperature with gentle shaking.
- Discard the fixing solution using aspiration, and rinse the gel with 300 mL of de-ionized water. Incubate for 10 min with gentle shaking. Repeat rinse two more times. The gel will swell slightly during rehydration.
- Discard the last of the water washes and add 300 mL of a freshly prepared 0.0005 % DTT solution. Incubate for 30 min with gentle shaking.
- Discard the DTT solution and add 300 mL of a 0.1 % $AgNO_3$ (freshly diluted from a 20 % stock, stored in a tightly closed, brown glass bottle at room temperature). Incubate for 30 min with gentle shaking.
- Discard the silver nitrate solution using aspiration, and rinse the gel 20 sec under a stream of de-ionized water. Do not allow the surface of the gel to dry, otherwise staining artifacts will occur.
- Prepare 450 mL of developing solution 3 % sodium carbonate, 0.05 % formalin (formalin is a 10 % formaldehyde solution in water; formaldehyde is usually obtained as a 37 % solution in water [check that the pH of the concentrated solution is greater than 4]). Add 150 mL of freshly prepared developing solution and incubate the gel at 24°C with gentle agitation for 30 sec. Decant and rinse the gel with water for 20 sec. Repeat once again.

- Add 150 mL of freshly prepared developing solution. Incubate the gel at 24°C with gentle agitation for 15-30 min or until the stained protein bands reach the desired intensity. Prolonged (>1 h) incubation leads to a high background of silver staining within the body of the gel.
- Terminate the reaction by adding 5 mL of a 72 % citric acid solution to each 150 mL of developing solution.
- Photograph the stained gel to make a permanent record.
- After staining, gels may be stored indefinitely in a sealed plastic bag containing a 20 % glycerol solution without any decrease in staining intensity.

Appendix 7.22 Amylose Determination using Concanavalin A:

Many of the properties of cereal starches that determine their suitability for particular end-uses are dependent upon their amylose/amylopectin ratios. These include gelatinization and gelation characteristics, solubility, the formation of resistant starch, the cooking and textural characteristics of whole grains. Thus, the measurement of the amylose content of starches is an important quality parameter for starch processing.

The Amylose/amylopectin assay kit (Megazyme) is based on the specific formation of amylopectin complexes with lectin concanavalin A (Con A). Under defined conditions of pH, temperature and ionic strength, Con A specifically complexes branched polysaccharides based on alpha-D-glucopyranosyl or alpha-D-mannopyranosyl units at multiple non-reducing end-groups with the formation of a precipitate. Thus, Con A effectively complexes the amylopectin component of starch but not the primarily linear amylose component.

The procedure described below is described by Gibson *et al.* (1997) and modifies a Con A method developed by Yun and Matheson (1990) by using an ethanol pre-treatment step to remove lipids prior to analysis. The procedure involves the following generalized steps. (1) Pure starch or cereals flours samples are completely dispersed by heating in dimethyl sulphoxide (DMSO). (2) Lipids are removed by precipitating the starch in ethanol and recovering the precipitated starch. Pre-treatment of the sample with ethanol has the added advantage of removing any soluble sugars in the sample that would otherwise interfere with the assay. (3) After dissolution of the precipitated sample in an acetate/salt solution, amylopectin is specifically precipitated by the addition of Con A and removed by centrifugation. (4) The amylose in an aliquot of the supernatant is enzymatically hydrolyzed to glucose, which is analyzed using glucose oxidase/peroxidase reagent. (5) The total starch in a separate aliquot of the acetate/salt solution is similarly hydrolyzed to glucose, using amyloglucosidase (hydrolyzes terminal 1,4-linked α-D-glucose residues successively from non-reducing ends of the chains with release of β-D-glucose) and α-amylase (random endohydrolysis of 1,4-α-glucosidic linkages in oligosaccharides and polysaccharides; reducing groups are reduced as α-D-glucose), and measured colorimetrically by glucose oxidase/peroxidase. The reactions involved are:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$2\ H_2O_2 + p\text{-Hydroxybenzoic acid} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4\ H_2O$$

(6) The concentration of amylose in the starch sample is estimated as the ratio of GOPOD absorbance at 510 nm of the supernatant of the Con A precipitated sample, to that of the total starch sample. (7) Repeated analyses of a set of samples yielded repeatability (within laboratory) relative standard deviations of < 5 % for pure starches and approx. 10 % for cereal flours. (Caution: DMSO is absorbed through the skin and can cause irritation to the skin and eyes. Avoid splashing and use in a fume cupboard. Clean up spills with excess water. Con A is harmful by inhalation and skin contact).

❖ **Starch Pre-treatment:**

- ✓ Accurately weigh starch or flour sample (20-25 mg ± 0.1 mg) into a 10 mL screw capped sample tube. Record the sample weight to the nearest 0.1 mg. Positive controls included, a high amylose reference corn starch sample (74.4 % amylose) provided in the kit and a normal corn starch (Sigma 4126; 27 % amylose and 73 % amylopectin).
- ✓ Add 1 mL of DMSO (BDH; Analytical grade; Cat. no. 10323) to the tube while gently stirring it at low speed on a vortex mixer. Cap the tube and heat the tube contents in a boiling water bath until the sample is completely dispersed (about 1 min). Ensure that no gelatinous lumps of starch are remaining.
- ✓ Vigorously mix the contents of the sealed tube at high speed on a vortex mixer, place the tube in a boiling water bath and heat it for 15 min, with intermittent high-speed stirring on a vortex mixer.
- ✓ Store the tube at 24°C for ~ 5 min and add 2 mL of 95 % ethanol with continuous stirring on a vortex mixer. Add a further 4 mL of ethanol, cap the tube, and invert to mix. A starch precipitate will form. Allow the tube to stand for 15 min.
- ✓ Centrifuge (2000 x g, 5 min), discard the supernatant, and drain the tubes on tissue paper for 10 min. Ensure that all the ethanol has drained. The pellet is used for subsequent amylose and starch determinations.
- ✓ Add 1 mL of DMSO (with gentle vortexing) to the starch pellet. Place the tube in a boiling water bath for 15 min and mix occasionally. Ensure that there are no gelatinous lumps.
- ✓ Prepare a 1 L stock of concentrated Con A solvent pH 6.4 (600 mM anhydrous sodium acetate; 3 M sodium chloride, 3 mM $CaCl_2 \cdot 2\ H_20$, 3 mM $MgCl_2 \cdot 6\ H_20$, and 4 mM $MnCl_2 \cdot 4\ H_20$) and store at 4°C. Prepare solution I as follows: add, with mixing, 2 mL of diluted Con A solvent (prepared by diluting 30 mL of concentrated Con A solvent to 100 mL with distilled water on the day of use) and quantitatively transfer the tube

contents (by repeated washing with Con A solvent) to a 25 mL volumetric flask. Dilute to volume with Con A solvent. Analysis of this solution should be performed within 60 min because the amylose will tend to retrograde and precipitate.

❖ **Con A Precipitation of Amylopectin and Determination of Amylose:**

- ✓ Transfer 1 mL of solution I to a 2 mL microfuge tube. Add 0.5 mL of Con A solution (3 mg/mL of Con A in diluted Con A solvent; stored in aliquots at -20°C), cap the tube and gently mix by repeated inversion. Avoid frothing of the sample.
- ✓ Allow the tube to stand for 1 h at 24°C (do not leave more than 2 h as the amylose will tend to retrograde). Centrifuge at 20 000 x g for 10 min in a microfuge tube at 20°C.
- ✓ Transfer 1 mL of the supernatant to a 15 mL centrifuge tube. Add 3 mL of 100 mM sodium acetate buffer, pH 4.5. This reduces the pH to 5. Mix the contents, lightly stopper (with a marble) and heat in boiling water bath for 5 min to denature the Con A.
- ✓ Place the tube in a water bath at 40°C and allow to equilibrate for 5 min. Add 0.1 mL of amyloglucosidase (EC 3.2.1.3)/alpha-amylase (EC 3.2.1.1) enzyme mixture (Dissolve 200 U of amyloglucosidase and 500 U of fungal α-amylase in 20 mL of 100 mM sodium acetate buffer; store at -20°C) and incubate at 40°C for 30 min. Centrifuge the tube at 2 000 x g for 5 min.
- ✓ To 1 mL aliquots of the supernatant add 4 mL GOPOD reagent (Dilute the entire contents of the glucose reagent buffer [1M potassium dihydrogen orthophosphate, 200 mM para-hydroxybenzoic acid, 0.4 % sodium azide] to 1 L with distilled water and use this to dissolve the glucose determination reagent; Reagent concentrations after dissolution in buffer are glucose oxidase 12 U/mL, peroxidase 0.65 U/mL, and 0.4 mM 4-aminoantipyrine; aliquot GOPOD reagent into aliquots of desired volume for storage for up to 1 year). Incubate at 40°C for 20 min.
- ✓ Incubate the Reagent Blank (add 1 mL of sodium acetate buffer to 4 mL of GOPOD Reagent) and the Glucose Controls (consists of 0.1 mL of glucose standard solution [1 mg/mL in 0.2 % (w/v) benzoic acid], 0.9 mL of sodium acetate buffer and 4 mL of GOPOD Reagent) at 40°C for 20 min. This value is not used in the calculation, however, it is determined to ensure that there are no problems with this part of the assay) concurrently.
- ✓ The absorbance at 510 nm for each sample, and the glucose controls are read against the reagent blank.

❖ **Determination of Total Starch:**

- ✓ Mix 0.5 mL of Solution I with 4 mL of 100 mM sodium acetate buffer, pH 4.5.
- ✓ Add 0.1 mL of amyloglucosidase/α-amylase solution and incubate the mixture at 40°C for 10 min.
- ✓ Transfer 1 mL aliquots (in duplicate) of this solution to glass test tubes and add 4 mL of GOPOD Reagent. Incubate at 40°C for 20 min. This incubation should be performed concurrently with samples and standards from the previous section.

❖ **Calculation of Amylose Concentration (%):**

The following equation was used to calculate % amylose for each sample:

% Amylose = Abs_{510} Con A Supernatant/Abs_{510} Total Starch Aliquot x 6.15/9.2 x 100

where Abs_{510} is the absorbance at 510 nm, 6.15 is the dilution factor for the Con A extract, and 9.2 is the dilution factor for the total starch extracts.

❖ **Comparison of Means for Quantitative Characters using an Unpaired t-Test:**

Standard deviation and standard error of the mean are two measurements of variability that are used in an unpaired t-test (Briggs and Knowles, 1967). The standard deviation, s_x, of a population may be estimated from a sample by the following formula:

$$s_x = \sqrt{\Sigma(x-\bar{x})^2/n-1}$$

All the deviations from the mean are squared, summed, and divided by a number that is one less than the number of measurements. Then the square root is taken of the quotient. The merit of the standard deviation is that it will tell rather precisely the range of variability of the population. It will say that in the range of the mean ± the standard deviation, 68 % of all measurements will be included. It will further say that in the range of the mean ● twice the standard deviation, 95 % of all measurements will be found.

If a second sample is drawn from a population it is very likely that its mean and standard deviation will differ slightly from those of the first sample. These differences are due to chance. Such means have a normal distribution, and the standard deviation of the mean is termed the standard error of the mean, $s_{\bar{x}}$. The standard error of the mean, may be determined from the standard deviation of the first sample or from the original measurements of the sample by the following formula:

$$s_{\bar{x}} = s_x/\sqrt{n}$$

where n is the number of measurements. This means that for 68 % of the samples drawn from the population, the mean will be in the range of ● the standard error of the mean. Means of variety yields, heights, seed weight, and other quantitative characters are usually expressed as the grand mean ± the standard error of the mean without explanation.

Significant difference between two means (Table 7.5) is determined by first calculating a standard error of a difference, s_d. The formula for this calculation is

$$s_d = \sqrt{s_{\bar{x}}^2} + \sqrt{s_y^2}$$

where $s_{\bar{x}}$ and s_y are standard errors of the means under comparison. The t-test is used to determine the significance of the difference. The t value is obtained by:

$$t = d/s_d$$

where d is the mean difference between the means. The significance of t is determined from a t table. Degrees of freedom = n-1. Compare the tabular t value with the calculated t value. If the calculated t value is greater than the tabular t value at a probability of 0.05 or 0.01, then chances of the samples having the same means are very low. They are significantly different.

Table 7.5 Amylose concentration data derived from triplicated starch samples of CDC Teal, Bai-Huo, Kanto 107, CDC Wx2, and CDC Wx6.

Treatment	Replication	Amylose Concentration (%)
CDC Teal	1	25.8
CDC Teal	2	27.5
CDC Teal	3	23.9
Mean		**25.7**
Bai-Huo	1	20.0
Bai-Huo	2	24.8
Bai-Huo	3	26.7
Mean		**23.8**
Kanto 107	1	13.6
Kanto 107	2	17.5
Kanto 107	3	17.7
Mean		**16.3**
CDC Wx2	1	1.0
CDC Wx2	2	4.3
CDC Wx2	3	6.8
Mean		**4.0**
CDC Wx6	1	12.1
CDC Wx6	2	15.3
CDC Wx6	3	14.6
Mean		**13.0**

Appendix 7.23 RNA Isolation Using Trizol Reagent:

Trizol reagent is a ready-to-use reagent for the isolation of total RNA from tissues. The reagent, a solution of guanidine isothiocyanate and phenol, maintains the integrity of the RNA during sample homogenization while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. The entire procedure can be completed in a couple of hours. Total RNA isolated by Trizol can be used for Northern blot analysis, dot blot hybridization, and poly (A)+ selection.

Trizol reagent facilitates isolation of a variety of RNA species of large and small molecular size. (Caution: Phenol reagent can cause severe burns within seconds of making contact with skin. When working with Trizol reagent use extreme caution by wearing gloves, face shield, and lab coat to avoid contact with skin, eyes, or clothing. Wash immediately with plenty of detergent and water to flush contaminated areas. Use in a chemical fume hood to avoid inhalation of reagent vapor). Unless otherwise noted the procedure is carried out at 24°C: RNA isolated from wheat endosperm tissue, separated on a 1.2 % agarose gel (size of DNA fragments separated is 0.4-7 kb), and stained with ethidium bromide, shows discrete bands of two predominant ribosomal RNA bands at ~ 5 kb (28S) and at ~ 2 kb (18S). The lower molecular weight RNA ~ 0.1-0.3 kb (t RNA, 5S) are not detected using a 1.2 % agarose gel. The isolated RNA is expected to have an $A_{260/280}$ ratio of 1.6-1.8, indicating that a reasonably pure RNA preparation has been obtained. Yields of total RNA per 100 mg of endosperm tissue at 5, 10 or 15 DPA were ~ 30 µg. The procedure is not recommended for isolating RNA from 20 and 25 DPA endosperm tissue due to poor RNA yields. The Trizol Reagent (Total RNA Isolation Reagent; GibcoBRL Cat. no. 15596) procedure is described below as outlined by the manufacturer:

- **Pre-cautions for Preventing RNase Contamination and Preparation of Materials used for RNA Purification** (Sambrook *et al.*, 1982).
  - ✓ Caution: Diethyl pyrocarbonate (DEPC) is a carcinogen thus handle with care under fume hood (DEPC is a strong but not an absolute inhibitor of RNases).
  - ✓ Gloves: Wear gloves and change them frequently.
  - ✓ Glassware, plastic ware, beakers, tubes, and other items: Individually wrapped plastic ware is RNase free. Clean glassware, spatulas, and mortar and pestles with detergent. Wrap them in foil and bake at 200°C for 8 h. Fill items (e.g., 50 mL centrifuge tubes and

glass bottle caps) with 0.1% DEPC water solution. Let stand for 2 h at 37°C, rinse several times with sterile water and autoclave for 15 min at 15 lb/sq.in. on liquid cycle to destroy remaining DEPC.

- ✓ Solutions: All solutions, except Tris solutions, should be prepared using RNase free glassware (DEPC treated), autoclaved water, and chemicals reserved for work with RNA that are handled with baked spatulas. Whenever possible, treat solutions with 0.1% DEPC for at least 12 h at 37°C then autoclave.
- ✓ Tris solutions: Tris buffers should be prepared with DEPC treated water and autoclaved. Dry chemicals should be purchased new and reserved only for RNA work (e.g., Tris) and weighed out with baked or flamed spatulas.
- ✓ Pipettes and microfuge tubes: Treat plastic ware containers with DEPC. Use gloves when preparing tips and tubes. Autoclave.

❖ **Homogenization:**

- ✓ Remove 100 mg of kernels from -80°C storage.
- ✓ Completely homogenize tissue samples in 1 mL of Trizol Reagent per 100 mg of kernels using a mortar and pestle cooled to 4°C. Insufficient homogenization of sample will reduce RNA yields.
- ✓ Following homogenization, remove insoluble material from the homogenate by centrifugation at 12 000 x g for 10 min at 5°C.
- ✓ Transfer the supernatant off the pellet.
- ✓ Equilibrate a 0.8 micron filter with 0.2 mL of Trizol reagent through a 3 cc syringe. Filter the supernatant through the equilibrated filter into a fresh microfuge tube.

❖ **Phase Separation:**

- ✓ Incubate the homogenized samples for 5 min at 24°C to permit the complete dissociation of nucleoprotein complexes.
- ✓ Add 0.2 mL of chloroform per 1 mL of Trizol Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 sec and incubate them at 24°C for 3 min.
- ✓ Centrifuge the samples at no more than 12 000 x g for 10 min at 5°C.
- ✓ Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an inter-phase, and a colorless upper phase. The volume of the aqueous phase is about 60 % of the volume of Trizol reagent used for homogenization.

❖ **RNA Precipitation:**

- ✓ Transfer of the aqueous phase to a fresh tube.
- ✓ Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 mL of isopropyl alcohol per 1 mL of Trizol reagent used for the initial homogenization. Incubate samples at 24°C for 10 min.
- ✓ Centrifuge at no more than 12 000 x g for 10 min at 5°C. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube.

❖ **RNA Wash:**

- ✓ Remove the supernatant. Wash the RNA pellet once with 75 % ethanol, adding at least 1 mL of 75 % ethanol per 1 mL of Trizol reagent used for the initial homogenization.
- ✓ Mix the sample by vortexing and centrifuge at no more than 7 500 x g for 5 min at 5°C.

❖ **Re-dissolving the RNA:**

- ✓ Vacuum dry the RNA pellet for 5-10 min. Do not over dry the pellet because this will greatly decrease its solubility.
- ✓ Dissolve the RNA pellet in 30 µL of RNase-free water and incubate at 60°C for 10 min to promote re-suspension of the RNA. Set tube on ice for 5 min.
- ✓ Centrifuge at 16 000 x g for 10 min to pellet gelatinized starch which co-purified with the RNA sample. Avoid the starch pellet in further work by using only the clear supernatant.

To determine the concentration of the resuspended RNA, dilute the sample 100 fold by adding 1 µL of sample to 99 µL of RNase-free water. Use RNase-free water to blank the DU Series 7400 Beckman Spectrophotometer at 260 nm. Quantification of RNA in this fashion is predicted on the fact that nucleic acids absorb UV light maximally at 260 nm. Place the diluted sample into a 100 µL quartz cuvette and read the absorbance (in optical densities) at 260 nm (A_{260}), 320 nm (A_{320}), and 280 nm (A_{280}). Determine the RNA concentration by using the following formula:

$$[\text{RNA}]\ \mu g/\mu L = (A_{260} - A_{320}) \times 44 \times D \times 1\ mL/1000\ \mu L$$

where A_{320} is the absorbance at 320 nm and is used as a background correction (Neither proteins nor nucleic acids absorb at this wavelength, so absorbance is due to other components. Subtraction of the absorbance at any of the other wavelengths corrects for the background

contribution); 44 is the extinction coefficient of RNA (an OD $_{260}$ [optical density] of 1 corresponds to approximately 44 µg/mL of single stranded RNA and DNA); D is the dilution factor (D = 100 in the above example); and 1 mL/1000 µL converts the [RNA] from to µg/mL to µg/µL. Determine the RNA yield by multiplying the concentration by the volume of the RNA. The A_{260} must be > 0.1 to give an accurate reading.

This calculation provides little information on the quality and purity of the sample, however in the presence of excess salt, contaminating proteins, and/or carryover organic solvents, the absorbance value can be skewed significantly. For these reasons, calculation of the 260:280 ratio (A_{260}-A_{320}/A_{280}-A_{320}) provides a reasonable estimate of the purity of the preparation. Absorbance at 280 nm is used for the detection of protein, based upon the presence of aromatic amino acids. A pure sample of RNA has a A_{260}/A_{280} ratio of 2 ± 0.05. If there is contamination with protein or phenol, the A_{260}/A_{280} ratio will be significantly less than the values given above, and accurate quantification of the amount of RNA will not be possible.

Appendix 7.24 Northern Blotting Protocol:

Methods are as described by Sambrook *et al.* (1982):

❖ **Electrophoresis of RNA Through Gels Containing Formaldehyde:**

- ✓ Caution: Formaldehyde vapors are toxic and solutions containing formaldehyde should be prepared in a chemical hood. DEPC is suspected to be a carcinogen and should be handled with care.
- ✓ Prepare 5X formaldehyde gel-running buffer pH 7.0 (0.1 M 3-N-morholino-propanesulfonic acid [MOPS] pH 7.0, 40 mM sodium acetate, and 5 mM EDTA pH 8.0).
- ✓ Electrophoresis tanks, gel tray and comb: Clean with detergent, rinse with water, dry with ethanol, and fill with a 3 % H_2O_2 solution. After 10 min at 24°C rinse with DEPC treated water.
- ✓ Prepare a 1.2 % gel by melting the appropriate amount of agarose in water, cooling it to 60°C, and adding 5X formaldehyde gel-running buffer and formaldehyde (at pH 4.0 or greater) to give final concentrations of 1X and 2.2 M, respectively. (One part of a stock 12.3 M formaldehyde solution should be diluted with 3.5 parts of agarose in water and 1.1 parts of 5X formaldehyde gel running buffer). Cast the gel in a chemical hood, and allow the gel to set for at least 30 min at 24°C. The single stranded nature of RNAs allow them to form secondary structures by intramolecular base pairing and must therefore be electrophoresed under denaturing conditions if good separations are expected. Denaturation is achieved by adding formaldehyde to the gel and loading buffer.
- ✓ Prepare the samples by mixing the following in a sterile microfuge tube: 4.5 µL of RNA (up to 30 µg), 2 µL of 5X formaldehyde gel-running buffer, 3.5 µL of formaldehyde, and 10 µL of formamide. Incubate the samples for 15 min at 65°C, and then chill them on ice. Centrifuge the samples for 5 sec to deposit all of the fluid in the bottom of the tubes. (Abundant mRNAs [0.1 % or more of the mRNA population can usually be detected by Northern analysis of 10-20 µg of total cellular RNA. For detection of rare RNAs, between 0.5 and 3 µg of poly $(A)^+$ RNA should be applied to each lane of the gel).
- ✓ Chill on ice and add 2 µL of sterile, DEPC-treated formaldehyde gel-loading buffer (50 % glycerol, 1 mM EDTA pH 8.0, and 0.25 % bromophenol blue).
- ✓ Before loading the samples, pre-run the gel for 5 min at 5 V/cm. Immediately load the samples into the lanes of the gel. As molecular weight markers, use RNAs of known size, for example, 18S and 28S rRNAs, The sizes of these RNAs are 6333 and 2366 nucleotides, respectively. Alternatively, mixtures of RNAs of known size can be

purchased from BRL. The markers are usually loaded into the outside lanes of the gel so that they can be cut from the gel after electrophoresis and stained with ethidium bromide. If possible, leave an empty lane between the markers and the samples that are to be transferred to a nylon membrane.

- ✓ Run the gel submerged in 1X formaldehyde gel-running buffer at 3-4 V/cm. After the buffer has been pulled into the gel, wash each well with 500 µL of running buffer. Constant recirculation of the buffer is not necessary, but after 1-2 h the buffer from each reservoir should be collected, mixed, and returned to the gel apparatus.
- ✓ At the end of the run (when the bromophenol blue has migrated approx. 8 cm), the gel may be stained with ethidium bromide (0.5 µg/mL in DEPC-treated water) for 30 min. Align a transparent ruler with the gel, and photograph the gel and ruler by ultraviolet illumination. Use the photograph to measure the distance from the loading well to each of the molecular weight standard marker bands of RNA. Plot the $\log_{10}$ of the size of the fragments of RNA against the distance migrated. Use the resulting curve to calculate the sizes of the RNA species detected by hybridization after transfer from the gel to a nylon membrane.
- ✓ Destain gel to remove formaldehyde and ethidium bromide by soaking in DEPC-treated 20X SSC buffer (3M NaCl, 0.3 M Na_3citrate) for 10 min. Decant water and repeat wash two more times.
- ✓ Set up capillary blot as described below.

❖ **Transfer of Denatured RNA to Nylon Membranes:**

- ✓ Transfer the gel to a glass baking dish, and trim away unused areas of the gel with a razor blade. Cut off the bottom left-hand corner of the gel; this serves to orient the gel during the succeeding operations.
- ✓ Place a piece of Whatman 3MM paper on a stack of glass plates to form a support that is longer and wider than the gel. Place the support inside a large baking dish. Fill the dish with 20X SSC until the level of the liquid reaches almost to the top of the support. When the 3MM paper on the top of the support is thoroughly wet, smooth out the air bubbles with a glass rod.
- ✓ Using a fresh scalpel, cut a piece of nylon membrane (Hybond N+; positively charged nylon membrane; Amersham) filter about the gel dimensions. Use gloves and blunt-ended forceps to handle the filter. A nylon membrane filter that has been touched by greasy hands will not wet.

- ✓ Float the membrane filter on the surface of a dish of de-ionized water until it wets completely from beneath, and then immerse the filter in 20X SSC for at least 5 min. Using a clean scalpel, cut a corner from the filter to match the corner cut from the gel.
- ✓ Place the gel on the support in an inverted position so that it is centered on the wet 3MM paper. Make sure that there are no air bubbles between the 3MM paper and the gel.
- ✓ Surround, but do not cover, the gel with saran wrap. This serves as a barrier to prevent liquid from flowing directly from the reservoir to paper towels placed on the top of the gel. If these towels are not precisely stacked, they tend to droop over the edge of the gel of the line of slots at the top of the gel. This type of short-circuiting is a major reason for inefficient transfer of RNA from the gel to the filter.
- ✓ Place the wet membrane on top of the gel so that the cut corners are aligned. One edge of the filter should just extend over the edge of the line of slots at the top of the gel. Do not move the filter once it has been applied to the surface of the gel. Make sure that there are no air bubbles between the filter and the gel.
- ✓ Wet two pieces of 3MM paper (cut to exactly the same size as the gel) in 2X SSC and place them on top of the wet membrane. Smooth out air bubbles with a glass rod.
- ✓ Cut a stack of paper towels (5-8 cm high) just smaller than the 3 MM papers. Place the towels on the 3 MM papers. Put a glass plate on top of the stack and weigh it down with a 500 g weight. The objective is to set up a flow of liquid from the reservoir through the gel and the membrane, so that RNA molecules are eluted from the gel and are deposited in the membrane.
- ✓ Allow transfer of RNA to proceed for 16-18 h. As the paper towels become wet, they should be replaced.
- ✓ Remove the paper towels and the 3MM papers above the gel. Turn over the gel and the membrane and lay them, gel side up, on a dry sheet of 3MM paper. Mark the position of the gel slots on the membrane with a pencil.
- ✓ Peel the gel from the membrane and discard it. Soak the membrane in 2X SSC for 5 min at 24°C. This removes any pieces of agarose sticking to the filter. To access the efficiency of transfer of RNA, the gel may be stained for 45 min in a solution of ethidium bromide (0.5 μg/mL in 0.1 M ammonium acetate) and examined by UV illumination.
- ✓ Place the dried filter for 1 h on a piece of 3MM paper. Wrap Hybond N+ in saran wrap and expose the side of the membrane carrying the RNA to a source of UV irradiation for 2-5 min. 312 nm wavelength is recommended. Dry membranes should be exposed to 0.15 J/cm^2. However, for maximum effect, it is important to make sure that the

membrane is not over irradiated. The aim is to form cross-links between a small fraction of the bases in the RNA and the positively charged amine groups on the surface of the membrane. Over-irradiation results in the covalent attachment of a higher proportion of thymines, with a consequent decrease in hybridization signal.

- ✓ If the membrane is not to be used immediately in hybridization experiments it should be wrapped loosely in aluminum foil and stored under vacuum at 24°C.

❖ **Calibration of UV Transilluminators:**

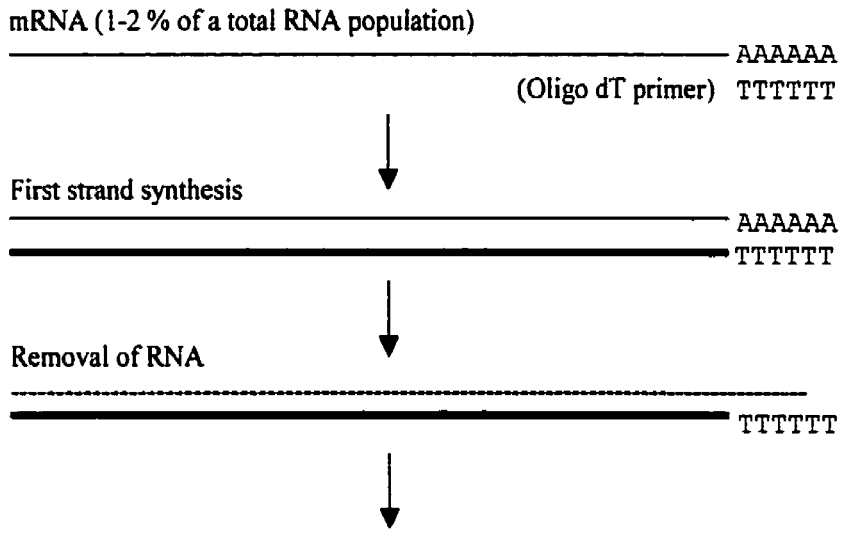
- ✓ Produce five or six identical strips of a blot of control DNA (e.g., restricted lambda) or RNA on Hybond N+. The type of blot will depend on the technique that the calibration is being used for. For standard Southern blots load 50 pg of lambda Hind III.
- ✓ Expose each blot DNA side up on the transilluminator for a different length of time, ranging from 30 sec to 10 min.
- ✓ Hybridize all the blots together with a suitable labeled probe.
- ✓ Following detection, the optimum UV exposure time will be indicated by selecting the filter showing the strongest signal.

❖ **Hybridization and Detection:**

- ✓ Conducted as described in appendix 7.3.

Appendix 7.25 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

The following protocol is as described by the Superscript pre-amplification system for first strand cDNA synthesis (Cat. no. 18089-011; GibcoBRL). The pre-amplification system is designed to synthesize first strand cDNA from purified poly (A)+ or total RNA. Following use of this system, target cDNA can be amplified with specific primers by PCR without intermediate organic extractions or ethanol precipitations. This process is summarized as follows:



The first strand cDNA synthesis reaction is catalyzed by Superscript II RNase H- Reverse Transcriptase (RT; catalyzes the synthesis of DNA in the 5'→3' direction with RNA template and DNA primer). This enzyme has been engineered to eliminate the RNase H activity that degrades mRNA during the first strand reaction. Use of the RNase H- RT results in greater full-length cDNA synthesis and higher yields of first strand cDNA than obtained with other RTs. This further improves the enzyme's ability to copy long RNA as compared to other RNase H- derivatives. Because Superscript II RT is not inhibited significantly by ribosomal and transfer RNA, it may be used effectively to synthesize first strand cDNA from a total RNA preparation. The enzyme exhibits increased thermal stability and may be used at temperatures up to 50°C.

Amplification of a target cDNA synthesized with this system requires priming with two specific oligonucleotides and Pfu DNA polymerase. The sensitivity of PCR amplification can be increased if RNase H is added after first strand synthesis is complete, yet before PCR to minimize

interference from the RNA template. Following amplification, RT-PCR products can be cloned into an appropriate vector for subsequent characterization procedures.

❖ **First Strand Synthesis using Oligo (dT):** This procedure is designed to convert poly A+ RNA into first strand cDNA:

- ✓ Mix and briefly centrifuge each component before use. Place on ice.
- ✓ Prepare the RNA/primer mixture in a sterile 0.5 mL tube:

Components	Sample	No RT Control
Total RNA (1 µg/µL)	1 µL	1 µL
Oligo $(dT)_{12\text{-}18\ mers}$ (0.5 µg/µL)	1 µL	1 µL
DEPC-treated water	10 µL	10 µL

- ✓ Incubate each sample at 70°C for 10 min and move it directly to 50°C. Using a thermal cycler simplifies the multiple temperature shifts in RT-PCR and can help prevent formation of secondary structure in RNA.
- ✓ Prepare the following reaction mixture, adding each component in the indicated order. For n samples plus one No RT control , prepare the reaction mix for n plus 2 reactions.

Components	Amount per reaction
10X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl)	2 µL
25 mM $MgCl_2$	2 µL
10 mM dNTP mix (10 mM each dATP, dCTP, dGTP,dTTP)	1 µL
0.1 M DTT (Protein reducing agent)	2 µL

- ✓ Pre-warm the 2X reaction mixture at 50°C before adding it to the primer and RNA. Add 7 µL of 2X reaction mixture to each RNA/primer mixture, mix gently, and collect by brief centrifugation.
- ✓ Add 1 µL of Superscript II RT (200 U/µL) to sample tubes, mix, and incubate for 12 min at each of the following temperatures: 50°C, 48°C, 46°C, 44°C, and 42°C. Do not add RT to No RT controls.
- ✓ Terminate the reactions at 70°C for 15 min. Chill on ice.

- ✓ Collect the reactions by brief centrifugation. Add 1 µL of RNase H (2 U/µL; an endonuclease that degrades the RNA portion of DNA-RNA hybrids) to each tube and incubate for 20 min at 37°C before proceeding to PCR amplification of the target cDNA.

❖ **PCR Amplification of the Target cDNA:**

The first strand cDNA may be amplified directly using PCR. Use only 10 % of the first strand reaction for PCR. Adding larger amounts of the first strand reaction may decrease the amount of product synthesized.

- ✓ Add the following to a 0.5 mL, thin-walled tube: two primer sets (MM2FP/GBSSR3 and GBSSF2/MMRTRP1) were used in the PCR screening.

Components	**Amount per reaction**
Distilled water	27.3 µL
Cloned Pfu DNA polymerase 10X reaction buffer	4 µL
dNTP mix (10 mM each)	1 µL
Primer #1 (5 pmoles/µL)	1.6 µL
Primer #2 (5 pmoles/µL)	1.6 µL
cDNA template	4 µL
Cloned Pfu DNA polymerase (2.5 U/µL)	0.5 µL
Total reaction volume	40 µL

- ✓ Gently mix and layer 2 drops of mineral oil over the reactions.
- ✓ Amplification conditions included an initial denaturation of 5 min at 94 °C (denatures the RNA/cDNA hybrid), followed by 20 cycles of 45 sec at 58 °C, 3 min at 72 °C, and 1 min at 94 °C.
- ✓ A 2 µL aliquot of the first PCR amplifications were used to conduct a second set of PCR amplifications as described above.
- ✓ Products from the second set of PCR amplification reactions were analyzed by electrophoresis on a 1.5 % agarose 1X TBE gel (100 V for 3 h) using ' or 40 µL of the reaction per lane.
- ✓ DNA fragments ranging from 1 to 1.6 kb in size were collectively cut from the gel and centrifuged at 16 000 x g for 30 min through siliconized glass wool.

- ✓ A 2-3 μL aliquot of blunt-ended DNA fragments (1 to 1.6 kb) was added to a 10-15 μL PCR reaction mixture containing: 1X Taq DNA polymerase buffer; 200 μM of dATP (100 mM dATP stock stored at -20°C); and 2.5 U Taq DNA polymerase (Boehringer Mannheim). The PCR reaction was incubated at 72°C for 4 h. The extension at 72°C using Taq DNA polymerase was used to facilitate the cloning of the blunt-ended PCR fragments derived.
- ✓ RT-PCR products were cloned into pCR2.1 T-vector for subsequent DNA sequencing characterization (Appendix 7.9).

Appendix 7.26 DNA Sequence Alignment of RT-PCR Derived cDNA Detected using MM2FP/GBSSR3. Sequences were aligned using the cluster method of the Megalign program (DNASTAR) using percent accepted mutation (PAM) 250 residue weight table. Nucleotides that are identical to the nucleotides of cDNA X57233 are indicated by the symbol '.'. Dashes (-) denote nucleotides absent in one of the sequences, but present in the others. Wheat cDNA derived from GenBank (Accession no. X57233; Clark *et al.*, 1991); wheat cDNA derived from GenBank (Accession no. Y16340); Teal 9-2 clone derived from CDC Teal; BH3-3 clone derived from Bai-Huo; Wx6-1 clone derived from CDC Wx6; and K4-2, K4-5, and K4-9 clones derived from Kanto 107 and CDC Wx2. The genomic DNA sequences, reported by Murai *et al.* (1999), of the *Wx-A1* gene encoding a 59 kD Wx-A1 protein (GenBank accession no. AB019622), the *Wx-B1* gene encoding a 58.8 kD Wx-B1 protein (accession no. AB019623), and the *Wx-D1* gene encoding a 58.9 kD Wx-D1 protein (accession no. AB019624) in hexaploid wheat are shown to differentiate intervening intron sequences. Primer sequences are presented at the extreme ends of clone sequences.

```
X57233        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Y16340        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Teal9-2       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
BH3-3         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-2          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-5          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-9          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Wx6-1         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
WX-A1 GENE    C C T G C G C G C G C C A T G G C G G C T C T G G T C A C G T C C C A 35
WX-B1 GENE    C C T G C G C G C G C G A T G G C G G C T C T G G T C A C G T C G C A 35
WX-D1 GENE    C C T G C G C G C G C C A T G G C G G C T C T G G T C A C G T C C C A 35

X57233        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Y16340        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Teal9-2       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
BH3-3         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-2          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-5          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-9          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Wx6-1         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
WX-A1 GENE    G C T C G C C A C C T C C G G C A C C G T C C T C A G C G T C A C C G 70
WX-B1 GENE    G C T C G C C A C C T C C G G C A C C G T C C T C G G C A T C A C C G 70
WX-D1 GENE    G C T C G C C A C C T C C G G C A C C G T C C T C G G C A T C A C C G 70

X57233        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Y16340        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Teal9-2       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
BH3-3         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-2          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-5          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-9          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Wx6-1         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
WX-A1 GENE    A C A G A T T C C G G C G T C C A G G T T T T C A G G G C C T G A G G 105
WX-B1 GENE    A C A G G T T C C G G C G T G C A G G T T T T C A G G G T G T G A G G 105
WX-D1 GENE    A C A G G T T C C G G C G T G C A G G T T T C C A G G G C G T G A G G 105

X57233        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Y16340        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Teal9-2       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
BH3-3         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-2          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-5          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
K4-9          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
Wx6-1         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1
WX-A1 GENE    C C C C G G A A C C C G G C G G A T G C G G C G C T C G G C A T G A G 140
WX-B1 GENE    C C C C G G A G C C C G G C A G A T G C G C C G C T C G G C A T G A G 140
WX-D1 GENE    C C C C G G A G C C C G G C G G A T G C G G C T C T C G G C A T G A G 140
```

```
X57233      -----------------------------------  1
Y16340      -----------------------------------  1
Teal9-2     -----------------------------------  1
BH3-3       -----------------------------------  1
K4-2        -----------------------------------  1
K4-5        -----------------------------------  1
K4-9        -----------------------------------  1
Wx6-1       -----------------------------------  1
WX-A1 GENE  GACTGTCGGAGCGAGCGCCGCCCCAAAGCAA---A  172
WX-B1 GENE  GACTACCGGAGCGAGCGCCGCCCCGAAGCAACAAA  175
WX-D1 GENE  GACCGTCGGAGCTAGCGCCGCCCCAACGCAA---A  172

X57233      -----------------------------------  1
Y16340      -----------------------------------  1
Teal9-2     -----------------------------------  1
BH3-3       -----------------------------------  1
K4-2        -----------------------------------  1
K4-5        -----------------------------------  1
K4-9        -----------------------------------  1
Wx6-1       -----------------------------------  1
WX-A1 GENE  GCAGGAAACCGCACCGATTCGACCGGCGGTGCCTC  207
WX-B1 GENE  GCCGGAAAGCGCACCGCGGACCCGGCGGTGCCTC  210
WX-D1 GENE  GCCGGAAAGCGCACCGCGGACCCGGCGGTGCCTC  207

X57233      -----------------------------CGGCAT  6
Y16340      -----------------------------......  6
Teal9-2     -----------------------------......  6
BH3-3       -----------------------------......  6
K4-2        -----------------------------......  6
K4-5        -----------------------------......  6
K4-9        -----------------------------......  6
Wx6-1       -----------------------------......  6
WX-A1 GENE  TCCATGGTGGTGCGCGCCACGGGCAGCGG......  242
WX-B1 GENE  TCCATGGTGGTGCGCGCCACGGGCAGCGC......  245
WX-D1 GENE  TCCATGGTGGTGCGCGCCACCGGCAGCGG......  242

X57233      GAACCTCGTGTTCGTCGGCGCCGAGATGGCCCCCT  41
Y16340      ..............................G....  41
Teal9-2     ..............................G....  41
BH3-3       ..............................G....  41
K4-2        ..............................G....  41
K4-5        ..............................G....  41
K4-9        ..............................G....  41
Wx6-1       ..............................G....  41
WX-A1 GENE  ..............................G....  277
WX-B1 GENE  ..............................G....  280
WX-D1 GENE  ..............................G....  277
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X57233      G G A G C A A G A C T G G C G G C C T C G G C G A C G T C C T C G G G  76
Y16340      . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . .  76
Teal9-2     . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . .  76
BH3-3       . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . .  76
K4-2        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  76
K4-5        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  76
K4-9        . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . .  76
Wx6-1       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  76
WX-A1 GENE  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  312
WX-B1 GENE  . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . .  315
WX-D1 GENE  . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . .  312
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X57233      G G C C T C C C C G C C G C C A T G G C C - - - - - - - - - - - - - -  98
Y16340      . . . . . C . A . . . . . . . . - - - - - - - - - - - - - -  98
Teal9-2     . . . . . C . A . . . . . . . . - - - - - - - - - - - - - -  98
BH3-3       . . . . . C . A . . . . . . . . - - - - - - - - - - - - - -  98
K4-2        . . . . . G G A C - - - - - - - - - - - - - - - - -  95
K4-5        . . . . . G G A C - - - - - - - - - - - - - - - - -  95
K4-9        . . . . . C . A . . . . . . . . - - - - - - - - - - - - - -  98
Wx6-1       . . . . . . . . . . . . . . . . - - - - - - - - - - - - - -  98
WX-A1 GENE  . . . . . . . . . . . . . . . . . . . . G T A A G C T T G C - - - -  344
WX-B1 GENE  . . . . . C . A . . . . . . . . . G T A A G C T A G C T A G C  350
WX-D1 GENE  . . . . . C . A . . . . . . . . . G T A A - - - - G C T A G A  343

X57233      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
Y16340      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
Teal9-2     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
BH3-3       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
K4-2        - - - - - - - - - - G C C T T C T T A T A A A T G T T T C T T C C T G  119
K4-5        - - - - - - - - - - G C C T T C T T A T A A A T G T T T C T T C C T G  119
K4-9        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
Wx6-1       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
WX-A1 GENE  - - - - G C C A C T G C C T T C T T A T A A A T G T T T C T T C C T G  374
WX-B1 GENE  T A G C A C C A C T G T C T T C T G A T A A - T G T T T C T T C T T G  384
WX-D1 GENE  C A G C A C C A C T G T C T T C T C A T A A - T G T T - C A T C T T G  376

X57233      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
Y16340      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
Teal9-2     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
BH3-3       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
K4-2        C A G C - - C A - - - - T G C C T G C C G T T A C A A - - - - - C G G  143
K4-5        C A G C - - C A - - - - T G C C T G C C G T T A C A A - - - - - C G G  143
K4-9        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
Wx6-1       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  98
WX-A1 GENE  C A G C - - C A - - - - T G C C T G C C G T T A C A A - - - - - C G G  398
WX-B1 GENE  C A G C - - C A G C C A T G C C T G C C A T T A C A A G T T T A C A A  417
WX-D1 GENE  C A G T T G C A G C C A T G C C T G C C G T T A C A A - - - - - C G G  406
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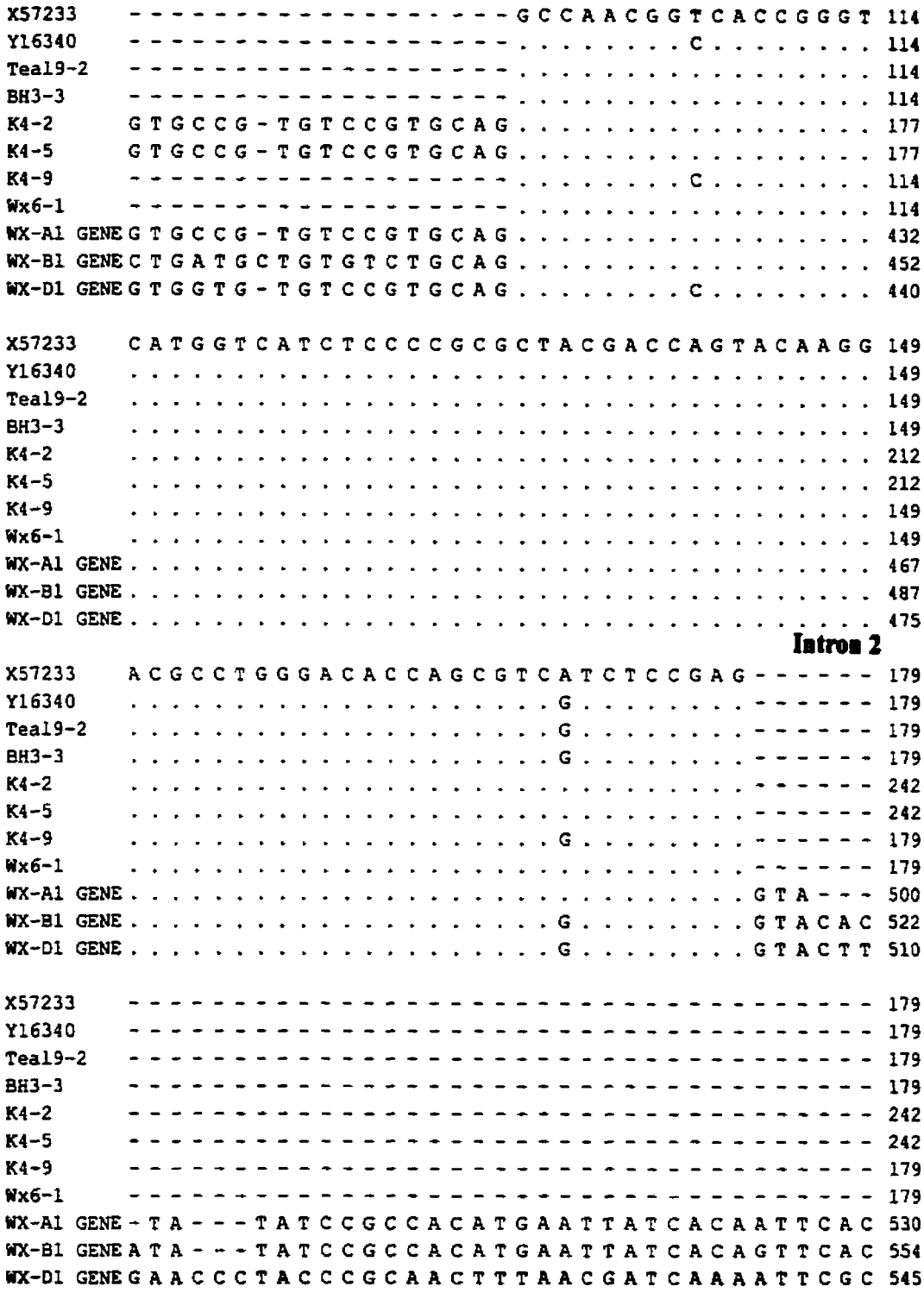
X57233 - G C C A A C G G T C A C C G G G T 114
Y16340 - . . . . . C 114
Teal9-2 - 114
BH3-3 - 114
K4-2 G T G C C G - T G T C C G T G C A G 177
K4-5 G T G C C G - T G T C C G T G C A G 177
K4-9 - . . . . . C 114
Wx6-1 - 114
WX-A1 GENE G T G C C G - T G T C C G T G C A G 432
WX-B1 GENE C T G A T G C T G T G T C T G C A G 452
WX-D1 GENE G T G G T G - T G T C C G T G C A G . . . . . C 440
X57233 C A T G G T C A T C T C C C C G C G C T A C G A C C A G T A C A A G G 149
Y16340 149
Teal9-2 149
BH3-3 149
K4-2 212
K4-5 212
K4-9 149
Wx6-1 149
WX-A1 GENE 467
WX-B1 GENE 487
WX-D1 GENE 475
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Y16340 . . . . . G . . . . . - - - - - - 179
Teal9-2 . . . . . G . . . . . - - - - - - 179
BH3-3 . . . . . G . . . . . - - - - - - 179
K4-2 . . . . . - - - - - - 242
K4-5 . . . . . - - - - - - 242
K4-9 . . . . . G . . . . . - - - - - - 179
Wx6-1 . . . . . - - - - - - 179
WX-A1 GENE . . . . . G T A - - - 500
WX-B1 GENE . . . . . G . . . . . G T A C A C 522
WX-D1 GENE . . . . . G . . . . . G T A C T T 510
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WX-A1 GENE - T A - - - T A T C C G C C A C A T G A A T T A T C A C A A T T C A C 530
WX-B1 GENE A T A - - - T A T C C G C C A C A T G A A T T A T C A C A G T T C A C 554
WX-D1 GENE G A A C C C T A C C C G C A A C T T T A A C G A T C A A A A T T C G C 545

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X57233       -----------------------------------  179
Y16340       -----------------------------------  179
Teal9-2      -----------------------------------  179
BH3-3        -----------------------------------  179
K4-2         -----------------------------------  242
K4-5         -----------------------------------  242
K4-9         -----------------------------------  179
Wx6-1        -----------------------------------  179
WX-A1 GENE   ATGCTCCTGCACATTTCTGCAAGACTTTACTGACT  565
WX-B1 GENE   ATGCTCCTGCACATTTCTGCAAGGTTCCACTCACC  589
WX-D1 GENE   ATGCTCCTGCACATTTCTGCAGGATCCTACTGACT  580

X57233       -------------------ATCAAGGTCGTTGACA  194
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Teal9-2      -------------------..........CG...G  194
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WX-A1 GENE   GGCT----GGATCTCGCAG................  596
WX-B1 GENE   GACT----GGATTTCACAG..........CG...G  620
WX-D1 GENE   GACTAACTGGATCTCGCAG................  615

X57233       GGTACGAGAGGGTGAGGTACTTCCACTGCTACAAG  229
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X57233       CGCGGGGTGGACCGCGTGTTCGTCGACCACCCGTG  264
Y16340       ...................................  264
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WX-A1 GENE   ...................................  666
WX-B1 GENE   ...................................  690
WX-D1 GENE   ...................................  685
```

Intron 3

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X57233          C T T C C T G G A G A A G - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Y16340          . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Teal9-2         . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
BH3-3           . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
K4-2            . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - 341
K4-5            . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - 341
K4-9            . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Wx6-1           . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
WX-A1 GENE      . . . . . G T G A C C G A T C G C - - T C G C C G - - 698
WX-B1 GENE      . . . . . G T G A C C A A T C G T C G T C G T C G A T 725
WX-D1 GENE      . . . . . G T G A C C G A T C G T C G T C G T G G A C 720

X57233          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Y16340          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Teal9-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
BH3-3           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
K4-2            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 341
K4-5            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 341
K4-9            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Wx6-1           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
WX-A1 GENE      - - - T C G A T C G A T C A A G C T A G C T C C T C G T C G T C T C A 729
WX-B1 GENE      C G A T C A A T C G A T C A A G C T A T C T T T T C G T C G T C T C A 760
WX-D1 GENE      C G A T C - - - - - - - - - A A G C T A G C T C T T C G T C G T C T C A 747

X57233          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Y16340          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Teal9-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
BH3-3           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
K4-2            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 341
K4-5            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 341
K4-9            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
Wx6-1           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 278
WX-A1 GENE      A C C C G C A T G G T G T T T G A T A A T T T C A G T G A G T C T T T 764
WX-B1 GENE      A C A T T C A T G G T G A T T G A - - - T T T G G G T G A G T C T T T 792
WX-D1 GENE      A C C T T G A T A G - G C A T G G T G A T T G A T T T C A G - - - T T 778

X57233          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - G T C C G G G G C A A 288
Y16340          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 288
Teal9-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 288
BH3-3           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 288
K4-2            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 351
K4-5            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 351
K4-9            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 288
Wx6-1           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 288
WX-A1 GENE      G C G T C T G C T G G T T A C A A T T T C C A G . . . . . 799
WX-B1 GENE      G T T T C T G C T G G T T G C A A T T T C C A G . . . . . 827
WX-D1 GENE      G T T T C T G C T G G T T G C A A T T T C C A G . . . . . 813
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X57233      G A C C A A G G A G A A G A T C T A T G G A C C C G A C G C C G G C A 323
Y16340      . . . . . C . . G . . . . . . . . . . . . 323
Teal9-2     . . . . . C . . G . . . . . . . . . . . . 323
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K4-9        . . . . . C . . G . . . . . . . . . . . . 323
Wx6-1       . . . . . 323
WX-A1 GENE  . . . . . 834
WX-B1 GENE  . . . . . C . . G . . . . . T . . . . . . . 862
WX-D1 GENE  . . . . . C . . G . . . . . . . . . . . . 848

X57233      C C G A C T A C G A G G A C A A C C A G C A G C G C T T C A G C C T T 358
Y16340      . G . . . . . 358
Teal9-2     . G . . . . . T A . . . . . G 358
BH3-3       . G . . . . . T A . . . . . G 358
K4-2        . . . . . 421
K4-5        . . . . . 421
K4-9        . G . . . . . 358
Wx6-1       . . . . . 358
WX-A1 GENE  . . . . . 869
WX-B1 GENE  . G . . . . . T A . . . . . G 897
WX-D1 GENE  . G . . . . . 883

X57233      C T C T G C C A G G C A G C A C T T G A G G T G C C C A G G A T C C T 393
Y16340      . . . . . G . . G . . G . . A . . . . . G . . . . . 393
Teal9-2     . . . . . G . . . . . C A . . . . . 393
BH3-3       . . . . . G . . . . . C A . . . . . 393
K4-2        . . . . . 456
K4-5        . . . . . 456
K4-9        . . . . . G . . G . . G . . A . . . . . G . . . . . 393
Wx6-1       . . . . . 393
WX-A1 GENE  . . . . . 904
WX-B1 GENE  . . . . . G . . . . . C A . . . . . 932
WX-D1 GENE  . . . . . G . . G . . G . . A . . . . . G . . . . . 918

X57233      C G A C C T C A A C A A C A A C C C A C A C T T T T C T G G A C C C T 428
Y16340      G A . . . . . G . . . . T . . . . . C T . . . . . G . . . . 428
Teal9-2     . . . . . T . . . . C . . C . . . . . 428
BH3-3       . . . . . T . . . . C . . C . . . . . 428
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Wx6-1       . . . . . 428
WX-A1 GENE  . . . . . 939
WX-B1 GENE  . . . . . T . . . . C . . C . . . . . 967
WX-D1 GENE  G A . . . . . G . . . . T . . . . . C T . . . . . G . . . . 953
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Intron 4

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X57233      A C G - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Y16340      . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Tea19-2     . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
BH3-3       . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
K4-2        . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  495
K4-5        . . . G T A A G A T C A A G A - - - - - - A C A A C T A G - - - - - -  515
K4-9        . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Wx6-1       . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
WX-A1 GENE  . . . G T A A G A T C A A G A - - - - - - A C A A C T A G - - - - - -  963
WX-B1 GENE  . . . G T A A G A T C A A C A - - - - - - A C A C C C A G C A G C T -  996
WX-D1 GENE  . . . G T A A G A T C A A G A T C A A G C A C G C C T A C T A G T T C  988

X57233      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Y16340      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Tea19-2     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
BH3-3       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
K4-2        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  495
K4-5        - - - - - A G T G T A T - - - - - - - - - - - - - - C T G A A G A A C  530
K4-9        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Wx6-1       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
WX-A1 GENE  - - - - - A G T G T A T - - - - - - - - - - - - - - C T G A A G A A C  978
WX-B1 GENE  - - A C T A G A G T G T - - - - - - - - - - - - - - C T G A A G A A C  1014
WX-D1 GENE  A A G C T A G A G T G T G T G T A A T C T G A A C T C T G A A G A A C  1023

X57233      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Y16340      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Tea19-2     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
BH3-3       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
K4-2        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  495
K4-5        T T G A T T T C T A C T T G A G A G C A C T G G A T G A T T A T C A T  565
K4-9        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Wx6-1       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
WX-A1 GENE  T T G A T T T C T A C T T G A G A G C A C T G G A T G A T T A T C A T  1013
WX-B1 GENE  T T G A T T T C T T C T T G A G A G C A C T G G A T G A T T A T C A T  1049
WX-D1 GENE  T T G A T A T T T T C T T G A G A G A G C T G G A T G A T C A C C A T  1058

X57233      - - - - - - - - - - - - - - - - - - - - - - C C A T G C T A T G C C G  444
Y16340      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Tea19-2     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
BH3-3       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
K4-2        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  495
K4-5        C T T C C T T - G T A T C T T G G T G C T G . . . . . . . . . . . . .  599
K4-9        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
Wx6-1       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  432
WX-A1 GENE  C T T C C T T - G T A T C T T G G T G C T G . . . . . . . . . . . . .  1047
WX-B1 GENE  C T T C C C T - G T G T C T T G G T G C T G . . . C . . C . . . . T A  1083
WX-D1 GENE  T T T T T T T T G T A T C T G G G T G C C G T . - - . T C G . C . . T  1091
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X57233      T G C C G T G C C G C G C C G C G C A G G G G A A G A C G T G G T G T 479
Y16340      - - - - - - - - - - - - - - - - - - - - - - - - G . . . . . . . . . . 446
Teal9-2     - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 446
BH3-3       - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 446
K4-2        - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 509
K4-5        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 634
K4-9        - - - - - - - - - - - - - - - - - - - - - - - - G . . . . . . . . . . 446
Wx6-1       - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . 446
WX-A1 GENE  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1082
WX-B1 GENE  . . . . . C . . . A . . . . . . . . . . . . . . . . . . . . . . . . . 1118
WX-D1 GENE  . . T T . - - - . . . . . G . . . . . 1123

X57233      T T G T G T G C A A C G A C T G G C A C A C G G G C C T T C T G G C C 514
Y16340      . C . . . . . T . . . . . 481
Teal9-2     . C . . . . . T . . . . . 481
BH3-3       . C . . . . . T . . . . . 481
K4-2        . . . . . 544
K4-5        . . . . . 669
K4-9        . C . . . . . T . . . . . 481
Wx6-1       . . . . . 481
WX-A1 GENE  . . . . . 1117
WX-B1 GENE  . C . . . . . 1153
WX-D1 GENE  . C . . . . . T . . . . . 1158

X57233      T G C T A C C T C A A G A G C A A C T A C C A G T C C A A T G G C A T 549
Y16340      . . . . . 516
Teal9-2     . . . . . G . . . . . 516
BH3-3       . . . . . G . . . . . 516
K4-2        . . . . . 579
K4-5        . . . . . 704
K4-9        . . . . . 516
Wx6-1       . . . . . 516
WX-A1 GENE  . . . . . 1152
WX-B1 GENE  . . . . . G . . . . . 1188
WX-D1 GENE  . . . . . 1193

                                  Intron 5

X57233      C T A T A G G A C G G C C A A G - - - - - - - - - - - - - - - - - - - 566
Y16340      . . . C . . . G . C . . A . . . - - - - - - - - - - - - - - - - - - - 533
Teal9-2     . . . . . - - - - - - - - - - - - - - - - - - - 533
BH3-3       . . . . . - - - - - - - - - - - - - - - - - - - 533
K4-2        . . . . . - - - - - - - - - - - - - - - - - - - 596
K4-5        . . . . . - - - - - - - - - - - - - - - - - - - 721
K4-9        . . . C . . . G . C . . A . . . - - - - - - - - - - - - - - - - - - - 533
Wx6-1       . . . . . - - - - - - - - - - - - - - - - - - - 533
WX-A1 GENE  . . . . . G T T T T G C A T C T T C T - - - G A 1184
WX-B1 GENE  . . . . . G T T T T G C A T C T T C T - - - C A 1220
WX-D1 GENE  . . . C . . . G . C . . A . . . G T T T T G C A T C T T C T T C T C A 1228
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X57233       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  566
Y16340       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
Teal9-2      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
BH3-3        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
K4-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  596
K4-5         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  721
K4-9         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
Wx6-1        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
WX-A1 GENE   A A C T T T A T A T T C G C T C T G C A T - - A T - - - C A - - - - -  1210
WX-B1 GENE   A A C T T T A T A T T C T C T C T G C A - - - - - - - - - - - - - - -  1241
WX-D1 GENE   A A C T A T A T A T C C T C T C T G C A T T C A T A T G C A T G C A T  1263

X57233       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  566
Y16340       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
Teal9-2      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
BH3-3        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
K4-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  596
K4-5         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  721
K4-9         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
Wx6-1        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
WX-A1 GENE   A T T T T G C G G T T C A T T C T G - - - - - - G C A - - - - - - - -  1231
WX-B1 GENE   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1241
WX-D1 GENE   A T C T T G C T C T T C A T T C T G A A A C A G G C A T A T C A A T T  1298

X57233       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  566
Y16340       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
Teal9-2      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
BH3-3        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
K4-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  596
K4-5         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  721
K4-9         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
Wx6-1        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  533
WX-A1 GENE   - - - - - - - - - - - - - - - G C C T G A A T T T T A C A T T G C A A  1250
WX-B1 GENE   - - - - - - - - - - - - - - - - - - - G A A T T T T A C A T T G C A A  1256
WX-D1 GENE   T T G C G G T T C A T T C T G G C C T G A A T T T T A C A T T G C A A  1333

X57233       - - - - - - - - - - - - - - - - - G T G G C A T T C T G C A T C C A C  583
Y16340       - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . .  550
Teal9-2      - - - - - - - - - - - - - - - - - . . A . . G . . . . . . . . . . . .  550
BH3-3        - - - - - - - - - - - - - - - - - . . A . . G . . . . . . . . . . . .  550
K4-2         - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . .  613
K4-5         - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . .  738
K4-9         - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . .  550
Wx6-1        - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . .  550
WX-A1 GENE   C T C C A T T T C A T G G C T A G . . . . . . . . . . . . . . . . . .  1285
WX-B1 GENE   C T T C A T T T C A T G T C C A G . . A . . G . . . . . . . . . . . .  1291
WX-D1 GENE   C T T C A T T T C A T G G C C A G . . . . . . . . . . . . . . . . . .  1368
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X57233     A A C A T C T C G T A C C A G G G C C G C T T C T C C T T C G A C G A 618
Y16340     . . . . . 585
Teal9-2    . . . . . T . . . . . 585
BH3-3      . . . . . T . . . . . 585
K4-2       . . . . . 648
K4-5       . . . . . 773
K4-9       . . . . . 585
Wx6-1      . . . . . 585
WX-A1 GENE . . . . . 1320
WX-B1 GENE . . . . . T . . . . . 1326
WX-D1 GENE . . . . . 1403

X57233     C T T C G C G C A G C T C A A C C T G C C T G A C A G G T T C A A G T 653
Y16340     . . . . . C . . . . . 620
Teal9-2    . . . . . C . . . . . 620
BH3-3      . . . . . C . . . . . 620
K4-2       . . . . . C . . . . . 683
K4-5       . . . . . G . . . . . C . . . . . 808
K4-9       . . . . . C . . . . . 620
Wx6-1      . . . . . C . . . . . 620
WX-A1 GENE . . . . . 1355
WX-B1 GENE . . . . . C . . . . . 1361
WX-D1 GENE . . . . . C . . . . . 1438

X57233     C G T C C T T C G A C T T C A T C G A C G G C T A C G A C A A G C C G 688
Y16340     . . . . . 655
Teal9-2    . . . . . 655
BH3-3      . . . . . 655
K4-2       . . . . . 718
K4-5       . . . . . 843
K4-9       . . . . . 655
Wx6-1      . . . . . 655
WX-A1 GENE . . . . . 1390
WX-B1 GENE . . . . . 1396
WX-D1 GENE . . . . . 1473

X57233     G T G G A G G G G C G C A A G A T C A A C T G G A T G A A G G C C G G 723
Y16340     . . . . . 690
Teal9-2    . . . . . 690
BH3-3      . . . . . 690
K4-2       . . . . . 753
K4-5       . . . . . 878
K4-9       . . . . . 690
Wx6-1      . . . . . 690
WX-A1 GENE . . . . . 1425
WX-B1 GENE . . . . . 1431
WX-D1 GENE . . . . . 1508
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X57233     G A T C C T G C A G G C C G A C A A G G T G C T G A C T G T G A G C C 758
Y16340     . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . 725
Teal9-2    . . . . . . . . . . . . . . . . . . . . . . . . . C . . G . . . . . . . 725
BH3-3      . . . . . . . . . . . . . . . . . . . . . . . . . C . . G . . . . . . . 725
K4-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 788
K4-5       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 913
K4-9       . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . 725
Wx6-1      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 725
WX-A1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1460
WX-B1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . C . . G . . . . . . . 1466
WX-D1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . 1543

X57233     C C T A C T A T G C T G A G G A G C T A A T C T C T G G C G A A G C C 793
Y16340     . . . . . . . C . . G . . . . . . . . C . . . . . . . . . . . . . . . 760
Teal9-2    . . . . . . . C . . G . . . . . . . . C . . . . . C . . . . . . . . . 760
BH3-3      . . . . . . . C . . G . . . . . . . . C . . . . . C . . . . . . . . . 760
K4-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 823
K4-5       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 948
K4-9       . . . . . . . C . . G . . . . . . . . C . . . . . . . . . . . . . . . 760
Wx6-1      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 760
WX-A1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1495
WX-B1 GENE . . . . . . . C . . G . . . . . . . . C . . . . . C . . . . . . . . . 1501
WX-D1 GENE . . . . . . . C . . G . . . . . . . . C . . . . . . . . . . . . . . . 1578

X57233     A G G G G C T G C G A G C T C G A C A A C A T C A T G C G C C T C A C 828
Y16340     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 795
Teal9-2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 795
BH3-3      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 795
K4-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 858
K4-5       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 983
K4-9       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 795
Wx6-1      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 795
WX-A1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1530
WX-B1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1536
WX-D1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1613

X57233     T G G G A T C A C C G G C A T C G T C A A C G G C A T G G A C G T C A 863
Y16340     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . T . 830
Teal9-2    G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 830
BH3-3      G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 830
K4-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 893
K4-5       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1018
K4-9       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . T . 830
Wx6-1      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 830
WX-A1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1565
WX-B1 GENE G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1571
WX-D1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . T . 1648
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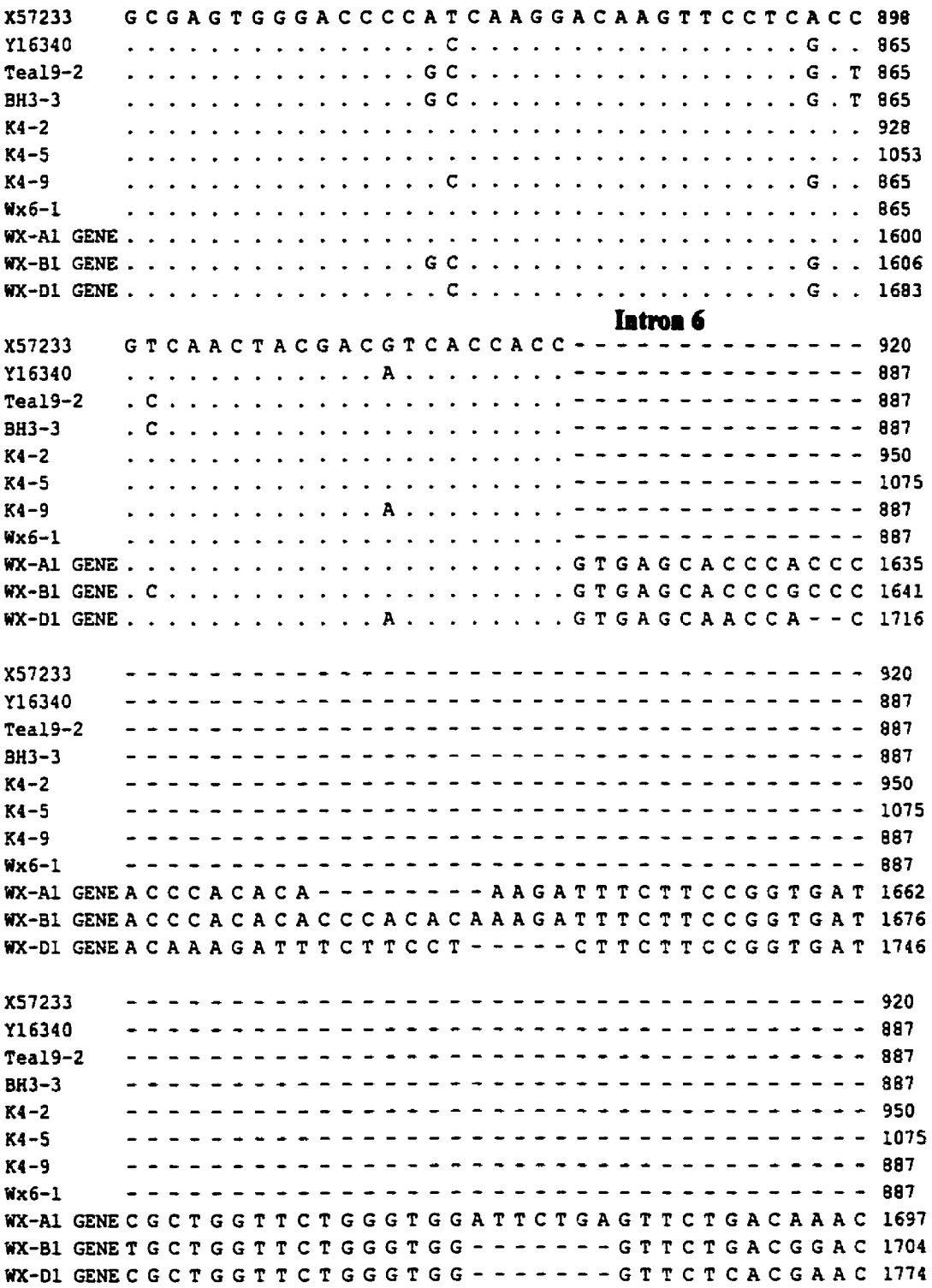
Intron 6

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X57233          - - - - - - - - - - - - - - - - G C G T T G G A G G G G A A G G C G C T  939
Y16340          - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . .  906
Teal9-2         - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . .  906
BH3-3           - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . .  906
K4-2            - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . .  969
K4-5            - - - - - - - - - - - - - - - - . . . . . . . . A . . . . . . . . . . .  1094
K4-9            - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . .  906
Wx6-1           - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . .  906
WX-A1 GENE      G A G G C A A A G T G A C A G . . . . . . . . . . . . . . . . . . . .  1732
WX-B1 GENE      G A G G C A A A G T G A C A G . . . . . . . . . . . . . . . . . . . .  1739
WX-D1 GENE      G A G G C A A A G T G A C A G . . . . . . . . . . . . . . . . . . . .  1809

X57233          G A A C A A G G A G G C G C T G C A G G C C G A G G T G G G G C T G C  974
Y16340          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  941
Teal9-2         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  941
BH3-3           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  941
K4-2            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1004
K4-5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1129
K4-9            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  941
Wx6-1           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  941
WX-A1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1767
WX-B1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1774
WX-D1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1844

X57233          C G G T G G A C C G G A A G G T G C C C C T G G T G G C G T T C A T C  1009
Y16340          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  976
Teal9-2         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . .  976
BH3-3           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . .  976
K4-2            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1039
K4-5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1164
K4-9            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  976
Wx6-1           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  976
WX-A1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1802
WX-B1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . .  1809
WX-D1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1879

X57233          G G C A G G C T G G A G G A G C A G A A G G G C C C C G A C G T G A T  1044
Y16340          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1011
Teal9-2         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1011
BH3-3           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1011
K4-2            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1074
K4-5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1199
K4-9            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1011
Wx6-1           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1011
WX-A1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1837
WX-B1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1844
WX-D1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1914
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X57233     G A T C G C C G C C A T C C C G G A G A T C G T G A A G G A G G A G G 1079
Y16340     . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . 1046
Teal9-2    . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . 1046
BH3-3      . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . 1046
K4-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1109
K4-5       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1234
K4-9       . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . 1046
Wx6-1      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1046
WX-A1 GENE . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1872
WX-B1 GENE . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . 1879
WX-D1 GENE . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . 1949

                                                    Intron 7
X57233     A C G T C C A G A T C G T T C T C C T G - - - - - - - - - - - - - - - - 1100
Y16340     . . . . . . . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - 1067
Teal9-2    . . . . . . . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - 1067
BH3-3      . . . . . . . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - 1067
K4-2       . . . . . . . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - 1130
K4-5       . . . . . . . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - 1255
K4-9       . . . . . . . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - 1067
Wx6-1      . . . . . . . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - 1067
WX-A1 GENE . . . . . . . . . . . . . . . . . . . . . . G T A C G - - A T C G A C C G 1905
WX-B1 GENE . . . . . . . . . . . . . . . . . . . . . . G T A C G T C A T C G A C C C 1914
WX-D1 GENE . . . . . . . . . . . . . . . . . . . . . . G T A C A T C A T C G A G C - 1984

X57233     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1100
Y16340     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
Teal9-2    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
BH3-3      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
K4-2       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1130
K4-5       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1255
K4-9       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
Wx6-1      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
WX-A1 GENE A C A T T G C T G A C C C G T T C A G G A A A A T C T C C T G A T A G 1940
WX-B1 GENE C A A C C G C - A A C C C G A C C G C C A - - - - T T G C T G A - A G 1943
WX-D1 GENE - - - C C G C - A A C C C G A C C G C C A - - - - T T G C T G A - A A 2009

X57233     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1100
Y16340     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
Teal9-2    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
BH3-3      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
K4-2       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1130
K4-5       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1255
K4-9       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
Wx6-1      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1067
WX-A1 GENE C T C G C C G T G G G G A T G G G T G G G T G A C T G A C T G A T C G 1975
WX-B1 GENE C T T - C A A T C A A G - C A G A C - - C T A A G - G A A T G A T C G 1973
WX-D1 GENE C T T - C G A T C A A G - C A G A C - - C T A A G - G A A T G A T C G 2039
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X57233          - - - - - - - - - - - - G G C A C C G G G A A G A A G A A G T T T G A 1122
Y16340          - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . C . . 1089
Teal9-2         - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . 1089
BH3-3           - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . 1089
K4-2            - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . 1152
K4-5            - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . 1277
K4-9            - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . C . . 1089
Wx6-1           - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . 1089
WX-A1 GENE      A A T G C A T T G C A G . . . . . . . . . . . . . . . . . . . . . . . . 2010
WX-B1 GENE      G A T G C A T T G C A G . . . . . . . . . . . . . . . . . . . . . . . . 2008
WX-D1 GENE      A A T G C A T T G C A G . . . . . . . . . . . . . . . . . . . . . C . . 2074

X57233          G C G G C T G C T C A A G A G C G T G G A G G A G A A G T T C C C G A 1157
Y16340          . . . . . . A . . . . . . . . . A . T . . . . . . . . A . . . . . . . 1124
Teal9-2         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1124
BH3-3           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1124
K4-2            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1187
K4-5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1312
K4-9            . . . . . . A . . . . . . . . . A . T . . . . . . . . A . . . . . . . 1124
Wx6-1           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1124
WX-A1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2045
WX-B1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2043
WX-D1 GENE      . . . . . . A . . . . . . . . . A . T . . . . . . . . A . . . . . . . 2109

X57233          C C A A G G T G A G G G C C G T G G T C A G G T T C A A C G C G C C G 1192
Y16340          G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1159
Teal9-2         G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1159
BH3-3           G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1159
K4-2            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1222
K4-5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1347
K4-9            G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1159
Wx6-1           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1159
WX-A1 GENE      . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . 2080
WX-B1 GENE      G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2078
WX-D1 GENE      G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2144

X57233          C T G G C T C A C C A G A T G A T G G C C G G C G C C G A C G T G C T 1227
Y16340          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1194
Teal9-2         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1194
BH3-3           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1194
K4-2            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1257
K4-5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1382
K4-9            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1194
Wx6-1           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1194
WX-A1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2115
WX-B1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2113
WX-D1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2179
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X57233          G G C G G T C A C C A G C C G C T T C G A G C C C T G C G G C C T C A 1262
Y16340          C . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1229
Teal9-2         C . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1229
BH3-3           C . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1229
K4-2            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1292
K4-5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1417
K4-9            C . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1229
Wx6-1           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1229
WX-A1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2150
WX-B1 GENE      C . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2148
WX-D1 GENE      C . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 2214

                                                                      Intron 8
X57233          T C C A G C T C C A G G G A A T G C G C T A C G G A A C G - - - - - - 1292
Y16340          . . . . . . . . . . . . . . G . . . . . . . . . . . . . . - - - - - - 1259
Teal9-2         . . . . . . . . . . . . . . G . . . . . . . . . . . . . . - - - - - - 1259
BH3-3           . . . . . . . . . . . . . . G . . . . . . . . . . . . . . - - - - - - 1259
K4-2            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . - - - - - - 1322
K4-5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . - - - - - - 1447
K4-9            . . . . . . . . . . . . . . G . . . . . . . . . . . . . . - - - - - - 1259
Wx6-1           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . - - - - - - 1259
WX-A1 GENE      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G T A A A C 2185
WX-B1 GENE      . . . . . . . . . . . . . . G . . . . . . . . . . . . . . G T A A A C 2183
WX-D1 GENE      . . . . . . . . . . . . . . G . . . . . . . . . . . . . . G T A A A C 2249

X57233          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1292
Y16340          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
Teal9-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
BH3-3           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
K4-2            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1322
K4-5            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1447
K4-9            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
Wx6-1           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
WX-A1 GENE      G C A T C C T C C T T C A G T C C T T C T T G C C A G T T C C T C A C 2220
WX-B1 GENE      G C C G C C T C C T - - - - - C C T T C C T G C C G A T T C C T T A T 2213
WX-D1 GENE      T T T T C C T T C T T G C C A A G T C C T T A C - - - T T C C T G A G 2281

X57233          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1292
Y16340          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
Teal9-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
BH3-3           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
K4-2            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1322
K4-5            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1447
K4-9            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
Wx6-1           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1259
WX-A1 GENE      C T C C T T T G C A T A T C C A T G G C C A T G A C C G A A G T T T C 2255
WX-B1 GENE      C T C C C C - G C G T A T C C A T G G C C A T G A C C G A A G T T T C 2247
WX-D1 GENE      C A A T C A T G - - - A G C C A T G C C C A T G A C C G A A G T T T C 2313
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X57233          - - - - - - - - - - - - - - - C C G T G C G C C T G C G C G T C G A C A 1312
Y16340          - - - - - - - - - - - - - - - . . . . . G . . . . . C . . C 1279
Teal9-2         - - - - - - - - - - - - - - - . . . . . G . . . . . C . . C 1279
BH3-3           - - - - - - - - - - - - - - - . . . . . G . . . . . C . . C 1279
K4-2            - - - - - - - - - - - - - - - . . . . . 1342
K4-5            - - - - - - - - - - - - - - - . . . . . 1467
K4-9            - - - - - - - - - - - - - - - . . . . . G . . . . . C . . C 1279
Wx6-1           - - - - - - - - - - - - - - - . . . . . 1279
WX-A1 GENE      T T T C A A A T T T T C A G . . . . . 2290
WX-B1 GENE      T T T C A A A T T T G C A G . . . . . G . . . . . C . . C 2282
WX-D1 GENE      T T C C A A A T T T T C A G . . . . . G . . . . . C . . C 2348

X57233          G G C G G G C T C G T C G A C A C T A T C G T G G A A G G C A A G A C 1347
Y16340          . . . . . T . . . . . G . . . . . G . . . . . 1314
Teal9-2         . . . . . G . . . . . G . . . . . 1314
BH3-3           . . . . . G . . . . . G . . . . . 1314
K4-2            . . . . . 1377
K4-5            . . . . . 1502
K4-9            . . . . . T . . . . . G . . . . . G . . . . . 1314
Wx6-1           . . . . . 1314
WX-A1 GENE      . . . . . 2325
WX-B1 GENE      . . . . . G . . . A . . . . G . . . . . 2317
WX-D1 GENE      . . . . . T . . . . . G . . . . . G . . . . . 2383

                                                                          Intron 9
X57233          C G G G T T C C A C A T G G G C C G C C T C A G C G T T G A C - - - - 1379
Y16340          . . . . . G . . . . . T . . C . . T - - - - 1346
Teal9-2         . . . . . C . . . - - - - 1346
BH3-3           . . . . . C . . . - - - - 1346
K4-2            . . . . . - - - - 1409
K4-5            . . . . . - - - - 1534
K4-9            . . . . . G . . . . . T . . C . . T - - - - 1346
Wx6-1           . . . . . - - - - 1346
WX-A1 GENE      . . . . . G T A T 2360
WX-B1 GENE      . . . . . C . . . G T A G 2352
WX-D1 GENE      . . . . . G . . . . . T . . C . . T G T A A 2418

X57233          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1379
Y16340          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1346
Teal9-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1346
BH3-3           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1346
K4-2            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1409
K4-5            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1534
K4-9            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1346
Wx6-1           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 1346
WX-A1 GENE      G C T C A T C G A T C C T C T T G T A T A C A T T C A T T C A T C T T 2395
WX-B1 GENE      G C T C G T C G A T C C - C T T G T G T A A A T T C - T T C A T T T T 2385
WX-D1 GENE      G T T C A T C A A T C - T C T T C A A T A A A T T C - T T C A T C T T 2451
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X57233       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1379
Y16340       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1346
Teal9-2      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1346
BH3-3        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1346
K4-2         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1409
K4-5         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1534
K4-9         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1346
Wx6-1        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1346
WX-A1 GENE   G T T C A T C A T G G C A G C T C A G A C A G A T C A T G A A G T G G  2430
WX-B1 GENE   G T T C A T C C T G G G A G C T C A G G C A G A T C A T G A A A T G G  2420
WX-D1 GENE   G T T C A T C C T G G G A G C T C A G G C A G A T C A T C A A A C G G  2486

X57233       - - - - - - - - - - - - - - - - - - - - - - - - - - T G C A A C G T G  1387
Y16340       - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . .  1354
Teal9-2      - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . .  1354
BH3-3        - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . .  1354
K4-2         - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . .  1417
K4-5         - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . .  1542
K4-9         - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . .  1354
Wx6-1        - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . . . . . .  1354
WX-A1 GENE   - T G C A C T T T T - C T T G T T G G T G G C C A G . . . . . . . . .  2463
WX-B1 GENE   - T T T C C T T T T T C C T C T T G G T G G C C A G . . . . . . . . .  2454
WX-D1 GENE   G T T T C C T T T T T C C T C T T G G T G G C C A G . . . . . . . . .  2521

X57233       G T G G A G C C G G C C G A C G T G A A G A A G G T G G T              1416
Y16340       . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              1383
Teal9-2      . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              1383
BH3-3        . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              1383
K4-2         . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              1446
K4-5         . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              1571
K4-9         . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              1383
Wx6-1        . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              1383
WX-A1 GENE   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C A C C A C  2498
WX-B1 GENE   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G A C C A C  2489
WX-D1 GENE   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G A C C A C  2556

X57233                                                                              1416
Y16340                                                                              1383
Teal9-2                                                                             1383
BH3-3                                                                               1383
K4-2                                                                                1446
K4-5                                                                                1571
K4-9                                                                                1383
Wx6-1                                                                               1383
WX-A1 GENE   C C T G A A G C G C G C C G T C A A G G T C G T C G G C A C G C C G G  2533
WX-B1 GENE   C C T G A A G C G C G C C G T C A A G G T C G T C G G C A C G C C A G  2524
WX-D1 GENE   C C T G A A G C G C G C C G T C A A G G T C G T C G G C A C G C C G G  2591
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X57233                                                                  1416
Y16340                                                                  1383
Teal9-2                                                                 1383
BH3-3                                                                   1383
K4-2                                                                    1446
K4-5                                                                    1571
K4-9                                                                    1383
Wx6-1                                                                   1383
WX-A1 GENE C G T A C C A T G A G A T G G T C A A G A A C T G C A T G A T A C A G 2568
WX-B1 GENE C C T A C C A T G A G A T G G T C A A G A A C T G C A T G A T C C A G 2559
WX-D1 GENE C A T A C C A T G A G A T G G T C A A G A A C T G C A T G A T A C A G 2626

X57233                                                                  1416
Y16340                                                                  1383
Teal9-2                                                                 1383
BH3-3                                                                   1383
K4-2                                                                    1446
K4-5                                                                    1571
K4-9                                                                    1383
Wx6-1                                                                   1383
WX-A1 GENE G A T C T C T C C T G G A A G G T A A G T C - G T C T C T G G T T C A 2602
WX-B1 GENE G A T C T C T C C T G G A A G G T A A G T C - G T C T C T G G T C T G 2593
WX-D1 GENE G A T C T C T C C T G G A A G G T A A G T C A G T C T C T G G T C T G 2661

X57233                                                                  1416
Y16340                                                                  1383
Teal9-2                                                                 1383
BH3-3                                                                   1383
K4-2                                                                    1446
K4-5                                                                    1571
K4-9                                                                    1383
Wx6-1                                                                   1383
WX-A1 GENE G T - - - - - A T G C A C T T C C T G G A A C A A C T A A G A G T G A 2632
WX-B1 GENE G T T T A G G A T G C A T T T T C C A G A A C A A C T A A G A G T T G 2628
WX-D1 GENE G T T T A G G A T G C A T T T T C C A G A A C A A C T A A G A G T T A 2696

X57233                                                                  1416
Y16340                                                                  1383
Teal9-2                                                                 1383
BH3-3                                                                   1383
K4-2                                                                    1446
K4-5                                                                    1571
K4-9                                                                    1383
Wx6-1                                                                   1383
WX-A1 GENE A G - - - - - - - - G G C - - - - - - - - - C G A T G T A T C C A T T 2650
WX-B1 GENE A G A C T A C A A T G G T G C T C G T G C T C G A T G C A T C C A T T 2663
WX-D1 GENE A G A C T A C A A T G G T G C T C T T G T T C G A T G T A T C C A T T 2731
```

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X57233                                                                1416
Y16340                                                                1383
Teal9-2                                                               1383
BH3-3                                                                 1383
K4-2                                                                  1446
K4-5                                                                  1571
K4-9                                                                  1383
Wx6-1                                                                 1383
WX-A1 GENE A A T G G T G G C T T G C G C A T A T G A T G C A G G G G C C T G C C 2685
WX-B1 GENE A A T G G T G G C T T G C G C A T A T G G T G C A G G G G C C A G C C 2698
WX-D1 GENE A A T G G T G G C T T G C G C A T A T G G T G C A G G G G C C A G C C 2766

X57233                                                                1416
Y16340                                                                1383
Teal9-2                                                               1383
BH3-3                                                                 1383
K4-2                                                                  1446
K4-5                                                                  1571
K4-9                                                                  1383
Wx6-1                                                                 1383
WX-A1 GENE A A G A A C T G G G A G G A C G T G C T T C T G G A A C T G G G G G T 2720
WX-B1 GENE A A G A A C T G G G A G G A C G T G C T T C T G G A A C T G G G G G T 2733
WX-D1 GENE A A G A A C T G G G A G G A C G T G C T T C T G G A A C T G G G T G T 2801

X57233                                                                1416
Y16340                                                                1383
Teal9-2                                                               1383
BH3-3                                                                 1383
K4-2                                                                  1446
K4-5                                                                  1571
K4-9                                                                  1383
Wx6-1                                                                 1383
WX-A1 GENE G G A G G G G A G C G A G C C G G G C A T C G T C G G C G A G G A G A 2755
WX-B1 GENE C G A G G G G A G C G A G C C A G G G G T C A T C G G C G A G G A G A 2768
WX-D1 GENE C G A G G G G A G C G A G C C G G G G G T C A T C G G C G A G G A G A 2836

X57233                                                                1416
Y16340                                                                1383
Teal9-2                                                               1383
BH3-3                                                                 1383
K4-2                                                                  1446
K4-5                                                                  1571
K4-9                                                                  1383
Wx6-1                                                                 1383
WX-A1 GENE T C G C G C C G C T C G C C C T G G A G A A C G T C G C C G C T C C C 2790
WX-B1 GENE T T G C G C C G C T C G C C A T G G A G A A C G T C G C C G C T C C C 2803
WX-D1 GENE T T G C G C C G C T C G C C A T G G A G A A C G T C G C C G C T C C C 2871
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X57233                                                    1416
Y16340                                                    1383
Tea19-2                                                   1383
BH3-3                                                     1383
K4-2                                                      1446
K4-5                                                      1571
K4-9                                                      1383
Wx6-1                                                     1383
WX-A1 GENE T G A A G A G A G A A A G A A                  2805
WX-B1 GENE T G A A G A G A G G A A A G A                  2818
WX-D1 GENE T G A A G A G A G A A A G A A                  2886
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Appendix 7.27 DNA Sequence Alignment of RT-PCR Derived cDNA Detected using GBSSF2/MMRTRP1. Sequences were aligned using the cluster method of the Megalign program (DNASTAR) using percent accepted mutation (PAM) 250 residue weight table. Nucleotides that are identical to the nucleotides of cDNA X57233 are indicated by the symbol '.'. . Dashes (-) denote nucleotides absent in one of the sequences, but present in the others. Wheat cDNA derived from GenBank (Accession no. X57233; Clark *et al.*, 1991); wheat cDNA derived from GenBank (Accession no. Y16340); FW 2-10 clone derived from CDC Wx2; BH13-3 and BH13-4 clones derived from Bai-Huo; and K14-9 clone derived from Kanto 107. MMRTRP1 is presented at the 3' end of the sequences.

```
X57233      C G A C G T G A A G A A G G T G G T C A C C A C C C T G A A G C G C G 35
Y16340      . . . . . G . . . . . 35
FW2-10      . . . . . 35
BH13-3      . . . . . G . . . . . 35
BH13-4      . . . . . 35
K14-9       . . . . . G . . . . . 35

X57233      C C G T C A A G G T C G T C G G C A C G C C G G C G T A C C A T G A G 70
Y16340      . . . . . A . . . . . 70
FW2-10      . . . . . 70
BH13-3      . . . . . A . . C . . . . . 70
BH13-4      . . . . . 70
K14-9       . . . . . A . . . . . 70

X57233      A T G G T C A A G A A C T G C A T G A T A C A G G A T C T C T C C T G 105
Y16340      . . . . . 105
FW2-10      . . . . . 105
BH13-3      . . . . . C . . . . . 105
BH13-4      . . . . . 105
K14-9       . . . . . 105

X57233      G A A G G G G C C T G C C A A G A A C T G G G A G G A C G T G C T T C 140
Y16340      . . . . . A . . . . . 140
FW2-10      . . . . . 140
BH13-3      . . . . . A . . . . . 140
BH13-4      . . . . . 140
K14-9       . . . . . A . . . . . 140

X57233      T G G A A C T G G G G G T G G A G G G G A G C G A G C C G G G C A T C 175
Y16340      . . . . . T . . C . . . . . G G . . 175
FW2-10      . . . . . 175
BH13-3      . . . . . C . . . . . A . . G G . . 175
BH13-4      . . . . . 175
K14-9       . . . . . T . . C . . . . . G G . . 175

X57233      G T C G G C G A G G A G A T C G C G C C G C T C G C C C T G G A G A A 210
Y16340      A . . . . . T . . . . . A . . . . . 210
FW2-10      . . . . . 210
BH13-3      A . . . . . T . . . . . A . . . . . 210
BH13-4      . . . . . 210
K14-9       A . . . . . T . . . . . A . . . . . 210

X57233      C G T C G C C G C T C C C T G A A G A G A G A A A G A A G A G G A G C 245
Y16340      . . . . . A . . . . A G 245
FW2-10      . . . . . 245
BH13-3      . . . . . G . . - - - - - . . A G . T 241
BH13-4      . . . . . 245
K14-9       . . . . . A . . . . A G 245

X57233      T T C T G G T G C A T G G A G C A T C C A T C C A A T C T G C A G G G 280
Y16340      . . . . . G . . . . . G . . . . . 280
FW2-10      . . . . . 280
BH13-3      . . T . . . . . T . . . . . - . . . T . . . . . 275
BH13-4      . . . . . 280
K14-9       . . . . . G . . . . . G . . . . . 280
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X57233      T T C T C G T A T G G G G A G A T A G C C G C T T G T T G T A G    312
Y16340      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    312
EW2-10      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    312
BH13-3      . . . C . . . G . . A . . . . . . . . . . . . . . . . . . . .    307
BH13-4      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    312
K14-9       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    312
```

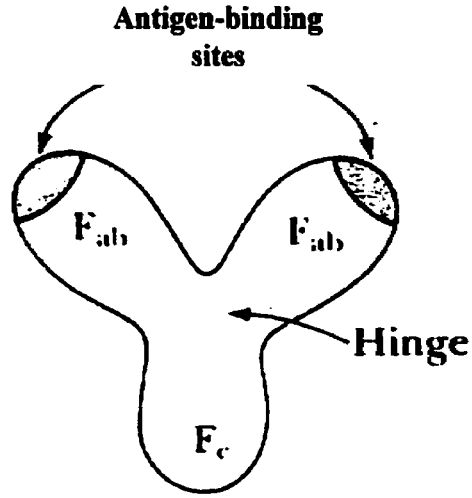
Appendix 7.28 Polyclonal Antibodies used in Western Blotting:

In response to the injection of a foreign substance into a higher animal (e.g., rabbit), an antibody (Ab) is produced that can react with the substance (Sambrook *et al.*, 1982). Antibodies are proteins found in the bloodstream and are part of a class of serum proteins known as immunoglobulins. Any substance (e.g., the denatured form of the 59-60 kD Wx-D1 protein isolated from the endosperm starch of wheat kernels) that can elicit antibody production is called an antigen (Ag). An antibody produced by exposure to an antigen has the important property of reacting specifically with the antigen that stimulated its production and not with most other antigens. Similarly, the antigen fails to react with any antibody other than that which it elicited.

In addition to the original antigen, denoted antigen A, there are other substances that react with a specific antibody, denoted antibody A (elicited by antigen A), though often with a somewhat lower efficiency. This weaker reaction is called a cross-reaction. One kind of cross-reaction is that which takes place when antigen B reacts partially with antibody A and antigen A with antibody B. Asymmetric cross-reactivity also occurs that is, antigen A reacts with antibody B, but antigen B does not react with antibody A. Cross-reactions occur when there is chemical similarity but not identity.

There are many types of antibody proteins found in serum. For the purpose of analytical immunological procedures, the most important family of antibodies is the immunoglobulin G or IgG (gamma globulin) class (e.g., IgG comprises ~ 85 % of the immunoglobulins in adult humans). These proteins, whose basic structure is shown schematically in Fig. 7.6 consists of three principal regions. Two of these regions are identical and are termed F_{ab} (F stands for fragment and ab for antigen-binding). The third section is called F_c. The central portion of an IgG molecule is a flexible region known as the hinge. Each F_{ab} branch contains a terminal antigen-binding site. Thus, each IgG molecule can bind two antigen molecules.

Since antibodies are produced in the bloodstream of an animal in response to the injection of a foreign substance then, the antibody can be obtained by bleeding an animal that has been repeatedly injected with the same antigen. Due to the specificity of the reaction of the Ag-Ab reaction, it is rarely necessary to isolate the specific antibody, or even the immunological fraction (i.e., IgG). Hence, in most immunological work, blood serum from which all cells have been removed by centrifugation is used. Serum known to contain a particular antibody is called antiserum.



7.6 Generalized Y shape of immunoglobulin G. Adapted from Sambrook *et al.* (1982)