Genetic control of Apigenin di-C-glycoside biosynthesis in bread wheat grain and their role as yellow pigments of Asian alkaline noodles

Submitted by

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This book is An Answer to Prayers of a Long list of Believers

I have been very blessed and loved with all of your spiritual supports, for His guidance and protections, and sincerely will not have enough to thank you all.....

May you all be blessed and loved, too

As I have been always,

yasmein

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Summary

Colour is an important determinant of quality and customer appeal for Asian noodles that are made from bread wheat (*Triticum aestivum* L.). The Asian noodle market represents approximately one third of wheat exports from Australia and as a consequence maintaining and improving colour for noodles is an important research and breeding objective. The focus of this project is yellow alkaline noodles (YAN) prepared using wheat flour and alkaline salts, sodium and potassium carbonate, and for which a bright yellow colour is desired. Xanthophylls, primarily lutein, and apigenin di-C-glycosides (ACGs) have been shown to be important components of this yellow colour. ACGs were of particular interest since, in contrast to lutein, the content in flour could be increased without adverse effects on colour of other end-products. There was little information either on the genetic variation for ACG content or the mechanism and genetic control of biosynthesis which was surprising in view of their putative role in a wide range of plant processes, food colour and flavour, and possibly human health.

The aims of this project were to provide new information on the role of ACGs in YAN colour and genetic regulation of their biosynthesis. To achieve this aims: genetic variation in grain ACG traits in bread wheat and related species was surveyed, the quantitative contribution of ACG to the yellow colour of YAN was determined and compared to lutein, QTL for ACG content and composition were located, and candidate genes associated with variation in ACG composition identified.

Substantial variation in both grain ACG content and the ratio, ACG1/ACG2, were identified within bread wheat cultivars and related species. Genotype controlled the

major portion of the variation. ACG content appeared to be a multigenic trait whereas variation in ACG1/ACG2 was associated with a limited number of chromosomes, in particular chromosomes 1B, 7B and 7D. In the absence of chromosome 7B (Chinese Spring 7B nullisomics) there was a substantial increase in ACG1/ACG2, i.e. a relative increase in the glucose-containing isomer, possibly indicating the presence of a *C*-glycosyltransferase on 7B with specificity for UDP-galactose. A similar phenotype observed in some wheat cultivars could be explained by a deletion or mutation of a gene controlling this enzyme. The results suggest that it should be possible to manipulate both ACG content and composition through breeding.

Only 30% of ACG (means 19.3μg/g) is recovered in flour, which contributed to 1 to 3 CIE b* units to the part of the yellow colour of yellow alkaline noodles (YAN) that develops specifically in the presence of alkali. The relatively low recovery of ACG in flour contrasts with the high recovery of lutein (90%, with means 1.011μg/g). Since the difference between white salted noodles (WSN) and YAN is approximately 6 b* units, this would indicate that another unidentified compound(s) is responsible for the difference. Potential for ACG0-based improvement of bread wheat cultivars for YAN yellowness is likely to be limited by the range of genetic variation, the location of ACG in grain tissues that are largely discarded during milling and the lack of correlation between grain and flour ACG content. Moreover, the observed variation in ACG recovery in small scale milling was not reflected in larger scale milling anticipated to better represent commercial practice. The improvement in flour recovery and the amount of ACG recovered in the flour were not significant and not enough to achieve the yellowness of commercial noodles. Selection that requires larger scale milling is costly, time consuming and not applicable to early generation screening. In this context,

further work on QTL associated with variation in ACG content and development of marker-assisted-selection would be very useful.

Addition of thirteen new markers to the QTL region for ACG trait on chromosome 7BS in a Sunco/Tasman doubled haploid population reduced the size of the QTL interval from 28.8cM to approximately 5.5cM. In this revised 7BS map, the major QTL for ACG1 and ACG2 content as well as ACG1/ACG2 ratio were detected within 4.7cM of SSR marker *Xwmc76*. The QTL region linked to *Xwmc76* was shown to be syntenic with a region in rice chromosome 6S between AP005387 and AP005761 and a region on *Brachypodium* chromosome 1. Based on these comparisons, the most likely candidate gene associated with variation in ACG composition appeared to be a glycosyltransferase. Alternate alleles at the 7BS QTL may be associated with amino acid changes within the *C*-glycosyltransferase that shift the substrate specificity from galactose (ACG2, Tasman) to glucose (ACG1, Sunco). Alternatively, based on a comparison of Chinese Spring nullisomic-tetrasomic lines where nullisomic 7B was associated with a phenotype similar to Sunco, it is possible that Sunco contains a null allele. Other candidate genes located on the same chromosome that could potentially be involved in ACG biosynthesis were identified and included a sugar transporter, which could determine the relative sizes of the available pools of UDP-glucose and UDP-galactose, an epimerase required for inter-conversion of these sugars, other glycosyltransferases and a flavone-2-hydroxylase (F2H) involved in the first committed step in the pathway to ACG.

Research approaches that could be used to validate the role of the candidate gene are discussed along with other options for improving the colour of wheat cultivars for the

YAN market. Options for utilizing ACG as yellow pigment of noodles might include incorporating the embryo or seed coat materials (pollard and bran) into the flour after milling and genetic modification of bread wheat to achieve ACG expression in the starchy endosperm.

Statement of Authorship

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Grace Yasmein Wijaya

November 2012

List of Publications

Wijaya GY, Asenstorfer R & Mares D 2010, 'Flavone-*C*-diglycosides and lutein in wheat grain: their contribution to the yellow colour of alkaline noodles', in: C Blanchard, D Pleming & H Taylor (eds), *Cereal 2009: Proceedings of the 59th Australian Cereals Chemistry Conference*, Wagga Wagga, New South Wales, Australia, 27-30 September 2009, Royal Australian Chemistry Institute, pp. 162-164.

Wijaya GY, Asenstorfer RA & Mares DJ (2010), 'Genetic control of *C*-glycosylation in the biosynthesis pathway of flavone-*C*-diglycosides, yellow colour substance of alkaline noodles, in bread wheat'. *Molecular Life- from discovery to Biotechnology*, *Ozbio 2010 (ASBMB conference in conjuction with the 12th IUBMB conference and FAOMB)*, Melbourne, 26 September-1 October 2010.

Wijaya GY, Mather DE, Brave M, Wu H, Walker AR, Chalmers KJ & Mares DJ 2012, 'QTL associated with variation in apigenin-*C*-diglycoside content and composition of bread wheat (*Triticum aestivum* L.) grain and the identification of candidate genes', *Molecular Mapping and Marker assisted Selection International Conference*, Vienna, 8-11 February 2012.

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List of Abbreviations

2-ODDs	2-oxoglutarate-dependent dioxygenases
ACG	apigenin di-*C*-glycosides
ACG1	apigenin-6*C*-arabinoside-8*C*-glucoside (isoschaftoside)
	apigenin-6C-glucoside-8*C*-arabinoside (schaftoside)
ACG2	apigenin-6*C*-arabinoside-8*C*-galactoside
	apigenin-6*C*-galactoside-8*C*-arabinoside
AFLP	amplified fragment length polymorphism
AMD	age-related macular degeneration
ANR	anthocyanidin reductase
ANS	anthocyanidin synthase
AS	aureusidin synthase
BAC library	bacterial artificial chromosome library
bHLH	basic/helix-loop-helix
Bz-W22	*O*-glycosyltransferases: UDP-glucosyl and glucuronosyl transferase
	of maize bronze alleles
cDNA	complimentary DNA, synthesise from mRNA
CHI	chalcone isomerase
CHS	chalcone synthase
CIE	Commission Internationale d'Eclairage
DArT	diversity arrays technology
DFR	dihydroflavonol-4-reductase
EBG	early biosynthetic genes
EST	expressed sequence tags

F2H	2*S*-flavanone 2-hydroxylase
F3H	flavanone-3β-hydroxylase
FCGT	flavonoid *C*-glycosyltranferase
FLS	flavonol synthase
FS1	flavone synthase 1
FS2	flavone synthase 2
GAT	UDP-glucuronic acid:flavonol-3-O-glucuronosyltransferase
gDNA	genomic DNA
GT	glycosyltransferases
HPLC	high performance liquid chromatography
IFS	isoflavones synthase
LAR	leucoanthocyanidin reductase
LBG	late biosynthetic genes
LOX	lipoxygenase
OMT	*O*-methyltransferases
OsCGT	*C*-glycosyltransferase of rice
PAC clone	P1 artificial chromosome clone
PPO	polyphenol oxidase
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
RT	rhamnosyl transferase
RT-PCR	reverse transcription polymerase chain reactions
SDR	short-chain dehydrogenase/reductases
SNP	single nucleotide polymorphism
SSR	simple sequence repeat

UDP-galactose	uridine 5′-diphosphogalactose
UDP-glucose	uridine 5′-diphosphoglucose
UFGT	UDP glycose:flavonoid-3-O-glycosyltransferase UDPG flavonol 3-*O*-glucosyl transferase
VvGT1	UDP-glucose:flavonoid 3-O-glycosyltransferase with ability to transfer UDP galactose in *Vitis vitifera*
VvGT5	UDP-glucuronic acid:flavonol-3-O-glucuronosyltransferase (GAT) of *Vitis Vitifera*
VvGT6	UDP-glucose/UDP-galactose:flavonol-3-O-glucosyltransferase/galactosyltransferase of *Vitis Vitifera*
WSN	white salted noodles
YAN	yellow alkaline noodles
Є-LCY	Є-cyclase

Chapter I: General Introduction

1.1 Background

Australia exports up to 80% of its wheat production which can represent approximately 18% of world wheat trade (Mohanty et al. 1995). Asian countries located in the South East Asia, East Asia, South Asia and Middle East are the biggest market for this wheat (Ahmadi-Esfahani & Stanmore 1997). In Japan, Korea and South East Asian countries, 1/3 of this imported wheat is used in the manufacture of noodles (Miskelly 1993, Wrigley 1994), with approximately 60% going into instant dried noodles, while the rest is used for making wet and dried white salted noodles (WSN) or yellow alkaline noodles (YAN).

There have been concerns with the wet noodle industry, especially with YAN, which is popular as street food in Asia. It is produced on a small scale by home industries scattered all over the country and widely distributed by traditional market food stalls, night markets, street food sellers, vending machines and travelling food sellers. There are no quality standards for the product, except the consumer preference for colour and taste. Governments in the region have tried to make a national standard, but have encountered difficulties because each noodle manufacturer has their own methods and recipes that have been part of their family tradition and inheritance.

YAN yellowness and the stability of this yellow colour is critical to consumer and market acceptance. To meet consumer preferences, noodle manufacturers may increase the concentration of alkaline solution, which is detrimental to the elasticity and pasting properties of the dough, or add colouring agents either natural or artificial which

influence the shelf-life of the noodles. To preserve the texture and lifetime of the noodles, chemicals such as borax (sodium tetraborate decahydrate) and formaldehyde have been known to be added to the dough (Khalik 2006) although these chemicals are potentially harmful for human health. There is an increasing market demand in the region for a reduction in the use of these additives and flour with inherent natural colour suitable for noodle production would have significant marketing advantages.

1.2 Knowledge Gap

Previous studies of flour and noodles identified some of the substances that give natural yellow colour to noodles such as the xanthophylls, primarily lutein, and apigenin di-*C*-glycosides (ACGs). Xanthophylls and their role in bread and noodle colour have been extensively studied. Increasing the xanthophyll content of wheat grain is one option for improving YAN colour however this would be likely to compromise the use of wheat flour in a wide range of other end-products, most of which are white to creamy in colour (Moss 1967). There is also an opportunity to increase the level of ACGs in wheat grain. However, in contrast with the numerous reports on xanthophyll content in grains, flour and noodles from a wide range of wheat varieties, no quantitative results have been reported for ACGs. Moreover, little is known regarding the genetic regulation of ACG biosynthesis in wheat grain. This project aims to provide new information on the role of ACGs in YAN colour and genetic regulation of their biosynthesis.

1.3 Structure of thesis

This thesis consists of 4 sections: the first section containing a general Introduction and a review of literature, the second section containing the general methods that were used throughout the study, the third section containing 4 experimental chapters and finally a

general discussion. The first experimental chapter discusses the genetic variation in ACG content and composition in bread wheat cultivars and species, and the possible chromosomal locations of the loci that control the trait genetically. A manuscript based on this chapter has been accepted for publication by the Journal of Cereal Science. In the second experimental chapter, the contribution of the ACGs to the yellow colour of noodles is discussed, included its comparison to that of lutein and the effect of 2 milling methods. The third chapter discusses the loci involved in the genetic control of ACG content and composition, where a major highly significant QTL region for the ACG traits at 7BS was fine mapped and the candidate genes were identified. Results from a preliminary study to optimise the existing PCR based methods to isolate the candidate genes are discussed in the fourth experimental chapter. Although the experiment has not yet come up with satisfactory results, the future direction for further application and optimisation of the methods are discussed.

Chapter II: Literature review

2.1 Asian noodles as one of the major end-products of Australian bread wheat

There are 3 types of wheat-based Asian noodles that differ in the nature of raw material used, the pH, processing methods and the organoleptic qualities. These are white salted noodles (WSN), Yellow alkaline noodles (YAN) and instant noodles (Corke & Bhattacharya 1999) (Table 2.1). In general, the basic ingredients for all of these noodles are flour of bread wheat (*Triticum aestivum*), water, salt, and/or alkaline salt solution (Miskelly 1996). These ingredients are mixed and then made into dough sheets by passing through a series of smooth steel rolls prior to cutting into noodle strands (Nagao 1996).

Table 2.1 Types of noodles based on nature of raw materials used, pH and organoleptic qualities (Corke & Bhattacharya 1999).

Types of Noodles	Ingredients	Noodle characteristics
White-salted noodles (WSN)	flour of low to medium protein level (8–10%), water (30–35%), sodium chloride (2-3%). pH 6.5–7	soft elastic texture and a smooth surface
Yellow-alkaline noodles (YAN)	hard wheat flour of medium protein content (10–12%), water (30–35%), alkaline salts, e.g., sodium carbonate, potassium carbonate; pH 9–11;	firm, chewy, springy texture and bright yellow appearance
Instant noodles	wheat flour of medium protein content (9–11%), water (30–35%), common salt or alkaline salts (2–3%), oil; pH 5.5–9	elastic, chewy texture

A flour characteristic that has an important influence on the choice by Asian countries for Australian wheats for their noodles is colour (Miskelly 1993). Flour with clean white colour is usually used for bakery products, while the bright white to creamy yellow flour is used for noodle manufacture (Ryan 2005). In general prime hard and Australian hard wheat grades, primarily from eastern Australia, are preferred for YAN whilst Australian Standard Noodle wheat cultivars (ASN), primarily from Western Australia, are used for WSN (Lush 2007, O'Brien et al. 2001).

Noodle consumers expect noodles to have a bright, clean appearance but there are variations in the preference for levels of yellowness between regions (Miskelly 1993). Differences in the yellow colour of flour between Australian wheat cultivars have been reported (Mares 1991) and there is an opportunity to breed cultivars with higher natural yellow flour colour especially for YAN.

2.2 Yellow colour of YAN

2.2.1 Natural Compounds that contribute to the yellow colour of YAN

2.2.1.1 Types of natural compounds that contributes to the yellow colour of YAN and their roles in plants and human health

There are 2 groups of natural compounds that have been reported to contribute to the yellow colour of YAN, xanthophylls and flavonoids (Mares & Mrva 2008). The xanthophylls in YAN dough are mainly in the form of lutein. Strong positive correlations have been reported between lutein content and the yellowness of noodles (Humphries et al. 2004). The flavonoids that are found in wheat grain and flour are mainly apigenin di-C-glycosides (ACGs) that are colourless at acid or neutral pH but turn yellow at the higher pH used in the preparation of YAN (Asenstorfer et al. 2006).

The presence of ACGs in wheat grain and flour is related to yellow colour of noodles but the correlation has not been quantified.

Xanthophylls ($C_{40}H_{54}(OH)_2$) (Figure 2.1) are produced by the carotenoid biosynthetic pathway with the immediate precursor being α–carotene (Howitt & Pogson 2006). Besides their role as a plant pigment, these substances also benefit human health. In flower petals, these substances provide a pale to deep yellow colour (Tanaka et al. 2008). In wheat endosperm, xanthophylls are present as lutein and give creamy colour to the flour and end-products (Kaneko & Oyanagi 1995). The accumulation of lutein in human retina is reported to contribute to a reduced incidence of retinal diseases, particularly age-related macular degeneration (AMD) (Schalch et al. 2007).

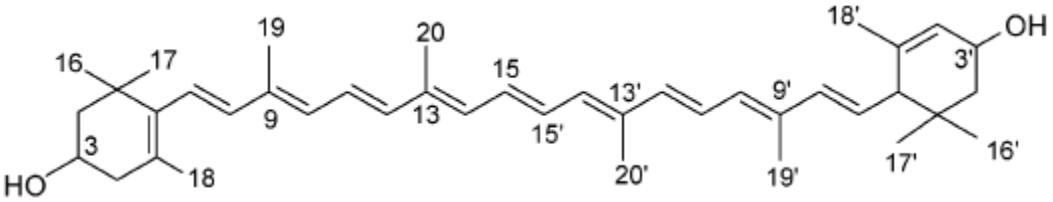

Figure 2.1 Structure of lutein (all-trans Lutein) (Abdel-Aal et al. 2007).

Apigenin di-*C*-glycosides (ACGs) in plants along with anthocyanins, flavonols, proanthocyanidins, aurones and phlobaphenes are flavonoids (Shirley 1998). Flavonoids represent a large and diverse class of secondary metabolites present in plants. The apigenin di-*C*-glycosides (ACGs) are a group of flavonoids that have been reported to contribute to the yellow colour of Asian alkaline noodles made from bread wheat,

Triticum aestivum L., flour (Asenstorfer et al. 2006). As a consequence, they are a potential target for wheat breeders attempting to develop new cultivars with improved noodle colour. An increase in the levels of these endogenous compounds would enable manufacturers to reduce the use of colour additives that are currently in common usage. In addition, ACGs also have reputed roles as bioactive compounds in herbal tea (McKay & Blumberg 2006) and curry powder (Satti et al. 2009), allelopathic compounds against parasitism and microbial infections (Hooper et al. 2010, Omar et al. 2011), feeding inhibitors of insect pests (Stevenson et al. 1996), as UV-protectants (Les & Sheridan 1990) and to have an-inflammatory and anticancer activity (Carvalho et al. 2010). However, despite their possible diverse and important roles for humans and agriculture, little is known of their biological functions in plants and the biochemical and genetic mechanisms that control their biosynthesis.

The ACGs are characterised by a common C6-C3-C6 flavone skeleton (Figure 2.2) with 3 carbons that are cyclized with an oxygen bridge between the 2 phenyl groups (Cavaliere et al. 2005). There are 2 groups of ACGs differentiated by the hexose sugar that is attached to the flavone A ring (Asenstorfer et al. 2006). The first group, ACG1, consists of a pair of Wesseley-Moser regioisomers with glucose at either the 6^{th} or 8^{th} carbon of the A ring of apigenin, while the second group, ACG2, consists of another pair of Wesseley-Moser regioisomers that have galactose rather than glucose attached to C6 or C8. In both ACGs, arabinose occupies the alternate carbon position not linked to the hexose sugar. The ACG1 regioisomers are diasterioisomers of ACG2.

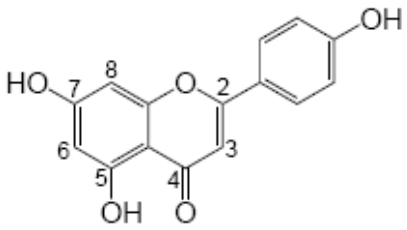

Figure 2.2 The general structure of flavones. ACGs are identified by sugar groups attached to 8-C and 6-C (Asenstorfer et al. 2006).

2.2.1.2 Contribution of xanthophylls and ACGs to the yellow colour of alkaline noodles

Xanthophylls and ACGs have different attributes in noodle dough. Xanthophylls are present at relatively low concentration in bread wheat and give colour ranging from white to pale yellow to noodle dough (Parker et al. 1998). On the other hand, the ACGs remain colourless in water or acid solution and give the yellow colour only after the alkaline solution is added during the making of YAN dough (Asenstorfer et al. 2006).

In wheat grain, these two compounds are located in different tissues which has a major impact on their recovery in flour as the result of milling (Miskelly 1996). The xanthophylls are located in embryo, grain coat and starchy endosperm; while the flavones are located in embryo and seed coat (Asenstorfer et al. 2006, Fortmann & Joyner 1978). The anatomy of wheat grain tissues are described in Figure 2.3.

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Figure 2.3 Anatomy of wheat grain tissues (Anon 1960). Embryo, endosperm and grain coat tissues comprise 2-3%, 81-84% and 14-16% of the grain respectively.

2.2.2 Factors influencing the measurement of the yellowness of noodles and the content of xanthophyll and ACG in wheat grain

Ideally, yellow alkaline noodles have a pale yellow colour (Kruger *et al.* 1994). High b* (Miskelly 1984) when measured according to the three dimension colour (L*-a*-b*) system proposed by the Commission Internationale d'Eclairage (CIE) (Wyszecki & Stiles 1982). The value of b* shows the position of the sample on the yellow-blue axis of the CIE 3-D colour space and a positive value indicates yellowness (Kruger *et al.* 1992). This yellowness is related to the sum of both carotenoid and flavonoid content (Mares et al. 1977 in Mares & Mrva 2001). Humphries et al. (2004) reported a positive correlation between b* value and carotenoid content in different groups of wheat grains and noted the influence of non-carotenoid substances in decreasing the b* value when grains from different wheat varieties are bulked.

Various methods have been published for the isolation, identification and quantification of xanthophylls and ACGs. Routine analysis of these two compounds in wheat grain, flour and noodle sheet can be performed by HPLC as described by Asenstorfer & Mares (2006), Breithaupt & Bamedi (2001), Fortmann & Joyner (1978), Soriano et al. (2007). However, in contrast with the availability of reports on xanthophyll content in wheat grain, flour and noodles, no quantitative analysis has been reported for ACGs in similar material.

Conditions of storage and timing are critical both for measurements of b* value and quantification of xanthophylls and ACGs of noodles and wheat grain. With noodles, in particular, the stability of the yellowness immediately after making the noodle sheet depends on the wheat variety used (Asenstorfer et al. 2006). This is influenced by the

condition of the samples in relation to the activities of the oxidative enzyme polyphenol oxidase (PPO), which result in darkening of noodles (Baik et al. 1995, Davies & Berzonsky 2003, Fuerst et al. 2006a, Mares & Mrva 2001) and lipoxygenase (LOX) that oxidises polyunsaturated fatty acids such as linoleic acid to hydroperoxides, which in turn are responsible for the oxidative degradation of yellow pigments such as lutein and β–carotene (Mares & Mrva 2008). The activities of PPO (darkening) masks the brightness and yellowness and is reflected the measurement of b* and L* values of noodles (Hessler et al. 2002, Mares & Mrva 2008, Pourcel et al. 2005). Similarly, LOX results in bleaching of yellow colour due to lutein and a reduction in b*.

In developing wheat grains, ACG in the embryo increased whilst that in the seed coat decreased with time after flowering (Ingram 2006, Rathjen 2006). The time of harvesting in terms of days post anthesis is therefore critical for analysis of ACG in grain and noodle sheets (Ingram 2006). In the case of xanthophyll measurement, the content of free lutein in wheat grain decreased slowly with time after harvest depending on the storage conditions, which determine the extent of esterification of lutein with free fatty acids (Soriano et al. 2007).

2.2.3 The amount, tissue location and composition of xanthophylls and apigenin di-C-glycosides in wheat grain and recovery in flour following milling

Variations has been found in total ACG content in grain of a limited number of wheat varieties (Asenstorfer et al. 2006). In addition, measurement of ACG content in wholemeal flour of several Australian wheat varieties shows that Sunco and Sunvale have significantly different proportions of the ACG1 and ACG2 when they are

compared with Angus, Avocet, and Canna (Asenstorfer et al. 2006). Amongst the Australian wheat varieties used by Rathjen (2006), SUN325 has the highest content of ACGs in the seed coat.

The yellow pigment content in the flour of different wheat varieties is influenced by milling (Adom et al. 2005, Chakraborty et al. 2003, Kuchel et al. 2006, O'Brien et al. 1993, Parker et al. 1999). ACGs are located in germ (Asenstorfer et al. 2006, Hatcher et al. 2008) and bran but are absent from the endosperm (Asenstorfer et al. 2006). Their availability in the flour that is used in noodle making, is the result of contamination by both germ and bran tissues during milling (Miskelly 1996). Milling can influence the b* value of flour and noodles even though the concentration of ACGs in the grain is the same. The amount of bran and germ and therefore ACGs in the flour depend on the physical structure and the milling qualities of the grain (Marshall et al. 1984, Oliver et al. 1993). Both are genetically controlled and influenced by environmental conditions (Graybosch et al. 2004, Oliver et al. 1993). On the other hand, xanthophylls are found in most parts of the wheat grain. The germ tissues contained the highest xanthophyll concentrations (Soriano et al. 2007), however, most of xanthophylls in wheat flour recovered from milling originates from the starchy endosperm that constitutes most of the flour (Marshall et al. 1984, Zhang et al. 2005). Therefore, in contrast to ACG their presence in flour is not dependent on contamination during milling.

2.3 Apigenin di-*C*-glycoside biosynthesis in plants

2.3.1 Flavonoid biosynthetic pathway

Apigenin di-*C*-glycosides (ACGs) in plants are produced by the flavonoid metabolic pathway. This pathway consists of a number of branches that produce distinct groups of

compounds (Quattrocchio et al. 2006) (Figure 2.4). There are several families of enzymes that function and interact with each other in the pathway, including the cytochrome P450 hydroxylases, the 2-oxoglutarate-dependent dioxygenases (2-ODDs), the short-chain dehydrogenase/reductases (SDR), the O-methyltransferases (OMT), and the glycosyltransferases (GT) (Himi & Noda 2006). The enzymes that function in the early part of the pathway such as chalcone synthase (CHS) and chalcone isomerase (CHI) are known to interact with those that initiate the branches of the pathway such as dihydroflavonol-4-reductase (DFR) and flavanone-3β-hydroxylase (F3H) (Hrazdina & Wagner 1985). Structurally, these proteins form a globular complex that is located at the cytoplasmic surface of endoplasmic reticulum, anchored to the membrane by other enzymes from the cytochrome P450 class (Burbulis & Shirley 1999, Ralston & Yu 2006, Shirley 2001).

Different levels of other flavonoid such as proanthocyanidins and phlobaphenes are known to relate with grain colour of rice and wheat (Himi et al. 2002, Sweeney et al. 2006). However, whether there is correlation between grain coat colour and ACG content in grain still needs to be confirmed. Although the alkaline noodles that are made from the flour of red grained wheat and white grained wheat show differences in b* value levels which might indicate differences in yellow pigment content in their grains (Seib *et al.* 2000), similar levels of apigenin di-*C*-glycosides were found in the grains of the red genotype R/W635 and white grained QT7475 (Rathjen 2006).

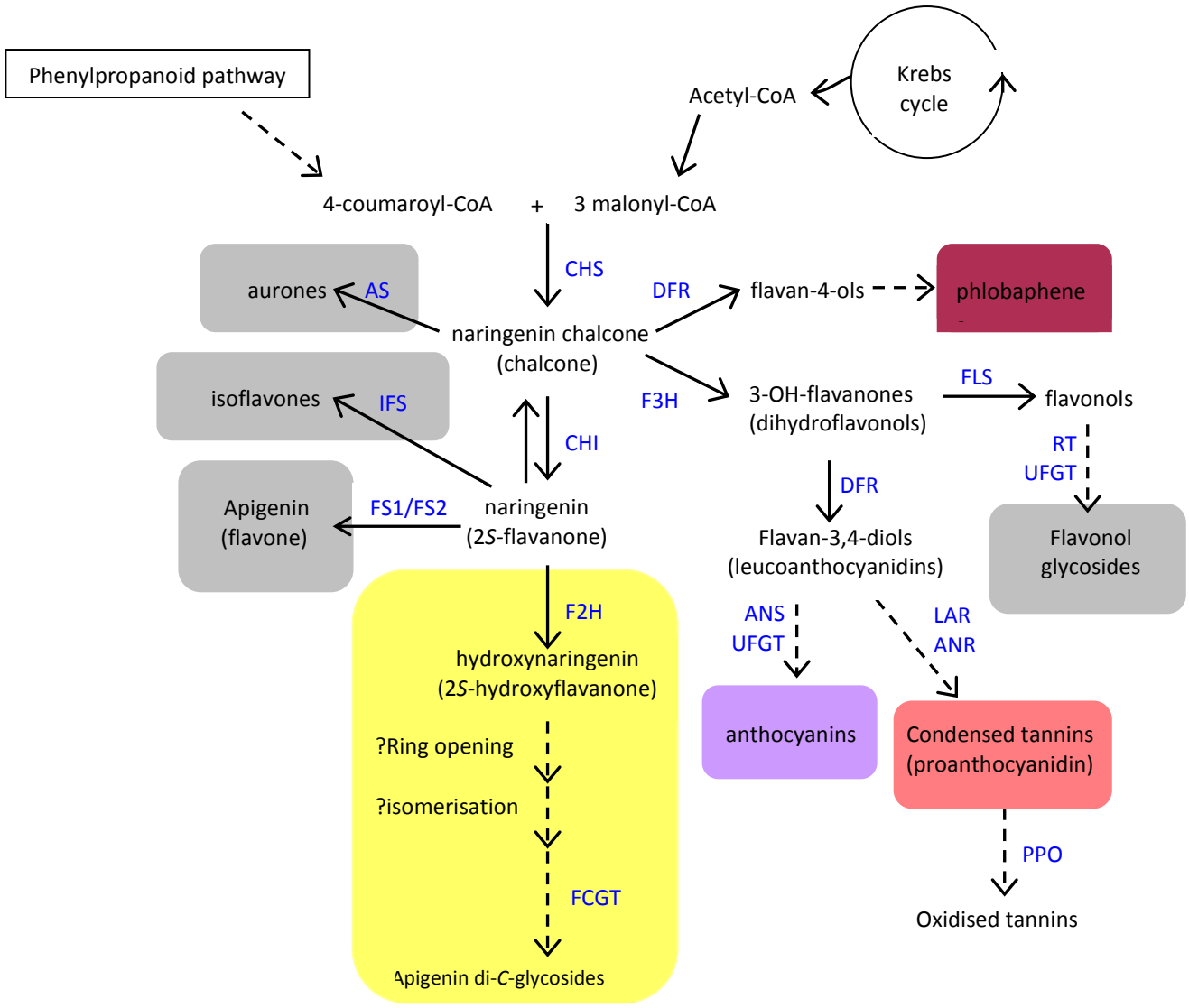

Figure 2.4 The flavonoid metabolic pathway (Asenstorfer et al. 2007, Ayabe & Akashi 2006, Cummins et al. 2006, Lepiniec et al. 2006, Martens & Forkmann 1999, Shirley 2001). The colours indicate branches of the pathway that lead to production of different types of flavonoid compounds. Enzymes that catalyse specific parts of the pathway are shown in blue. Those that function in the first part of the flavonoid pathway: CHS (chalcone synthase) and CHI (chalcone isomerase). Those are proposed to function in flavone-C-diglycoside biosynthesis: F2H (2S-flavanone 2-hydroxylase) and FCGT (flavonoid C-glycosyltranferase), while those that function in flavone biosynthesis: FS1 (flavone synthase 1) and FS2 (flavone synthase 2). Other branches leading to anthocyanin biosynthesis: F3H (flavanone-3β-hydroxylase), DFR (dihydroflavonol-4-reductase), ANS (anthocyanidin synthase), UFGT (UDP glycose:flavonoid-3-O-glycosyltransferase), for flavonol biosynthesis: FLS (flavonol synthase); RT (rhamnosyl transferase), for aurones biosynthesis: AS (aureusidin synthase), for isoflavones biosynthesis: IFS (isoflavones synthase), for proanthocyanidin biosynthesis: LAR (leucoanthocyanidin reductase) and ANR anthocyanidin reductase.

2.3.2 Pathway leading to apigenin di-C-glycoside biosynthesis in wheat grain

By comparison with the biosynthesis of anthocyanins, phlobaphenes, and proanthocyanidins, the part of the flavonoid pathway that leads to the biosynthesis of ACGs in wheat grain is still only poorly understood. However, based on other studies in dicotyledonous plants and cereals such as rice and maize, there are several candidate enzymes that might be involved in ACG biosynthesis (Table 2.2). Two types of flavone biosynthesis enzymes, flavone synthase 1 (FS1) and flavone synthase 2 (FS2), have been identified in *Petroselinum hortense*, rice and *Gerbera hybrida* (Lee et al. 2008, Martens & Forkmann 1998, 1999, Martens et al. 2001, Zhang et al. 2007). These two enzymes have different mechanisms for biosynthesis of flavone from flavanone: FS1 directly converts flavanone into flavone (Martens et al. 2001) while FS2 performs the conversion via the formation of an intermediate compound, (*2S*)-hydroxyflavanone (Akashi et al. 1999).

Table 2.2 Enzymes involved in flavone biosynthesis.

Enzymes	Un-abbreviated names	Plant Source	Sequence of the pathway regulated	Reference
FNSI	flavone synthase I	Parsley (*Petroselinum hortense*) cv. Italian Giant Rice	direct convertion of (2S)-naringenin to apigenin (flavanones to flavones)	Martens et al., 2001 Lee et al., 2008
FNSII (CYP93B2)	flavone synthase II (cythochrome 450 93B2)	*Gerbera hybrida*	convertion of (2S)-naringenin to apigenin (flavanones to flavones) through the formation of (2S)-hydroxynaringenin (hydroxyflavanones)	Martens and Forkmann, 1999
MtFNSII-1 MtFNSII-2	*Medicago truncatula* flavone synthase-1 *Medicago truncatula* flavone synthase-2	*Medicago truncatula*	conversion of flavanones to (2S)-hydroxyflavanones	Zhang et al., 2007
F2H (CYP93B1)	flavanone 2-hydroxylase (cythochrome 450 93B1)	Licorice (*Glycyrrhiza echinata* L.); Fabaceae Snapdragon (*Antirrhinum majus*) Torenia (*Torenia fournieri*)	conversion of flavanones to (2S)-hydroxyflavanones	Akashi et al., 1998 Akashi et al., 1999
FCGT	flavonoid *C* glycosyltranferase	Wheat (*Triticum aestivum* L.) Buck-wheat (*Fagopyrum esculentum*)	addition of 2 glycosyl groups to the hydroxyflavanones, after hemiketal ring opening	Cummins et al., 2006 Kerscher and Franz, 1987

Later in the pathway, glycosylation possibly catalysed by a flavonoid-*C*-glycosyltransferase (FCGT) is suggested to occur at 6-C and 8-C of apigenin moiety (Kerscher & Franz 1988). As naringenin, naringenin chalcone or apigenin do not appear to be substrates for glycosylation (Kerscher & Franz 1988), it is suggested that the ACG biosynthesis proceeds through a different pathway than those initiated by FS1 and FS2. The addition of sugar molecules is suggested to take place after a member of cytochrome P450 superfamily that has very similar amino acid sequences with FS2 of *Gerbera* (CYP93B2), CYP93B1 (2S-flavanone 2-hydroxylase (F2H)), which converts flavanone into hydroxyflavanone by monooxygenation of the flavanones as has been observed in *Glycyrrhiza echinata*, *Anthirrhinum majus*, *Torenia fournieri* and *Medicago truncatula* (Akashi et al. 1998, Zhang et al. 2007).

The involvement of hydroxyflavanone as substrate in biosynthesis of ACG by a glycosyltransferase was also observed by Cummins et al. (2006). A change in the ACG content of 7 days old wheat seedlings treated with cloquintocet mexyl herbicide safeners was reported to be correlated with the increase of *C*-glycosyltransferase activity towards 2-hydroxynaringenin. Based on this fact and other previous studies (Asenstorfer et al. 2006, Brazier-Hicks et al. 2009, Hamilton et al. 2009, Kerscher & Franz 1987), a novel branch of the flavonoid biosynthetic pathway leading to ACGs in wheat grain is proposed in Figure 2.5 The initial step involves conversion of *2S*-naringenin flavanone to the hemiketal structure of the flavanone by flavanone *2*-hydroxylase (F2H) (Du et al. 2010, Du et al. 2009). Prior to the glycosylation of 2-hydroxyflavanone, the hemiketal ring is suggested to open, which activates 6-*C* and 8-*C* to nucleophilic attack (Asenstorfer et al. 2006). This was supported by the identification of the 2 sets of Wesley-Moser isomers of ACGs in wheat embryo tissues. One set

contains arabinose and glucose at 6-*C* and 8-*C* position whilst in the other set, glucose is replaced with galactose. The reactions that follow the *C*-glycosylation are similar for all the ACG isomers, with cyclisation, not requiring an enzyme, and water loss involving a dehydratase (Grotewold et al. 1998, Shirley 2001). The hexose *C*-glycosylation is possibly a key reaction determining which diasterioisomer, ACG1 or ACG2, is formed. Brazier-Hicks et al. (2009) suggested that there was a specific *C*-glucosyltransferase for the addition of UDP-glucose to *2S*-hydroxynaringenin or its hemiketal in rice.

The glycosyltransferase family has a very diverse protein sequences. Those reported in plants such as UDP glycose:flavonoid-3-O-glycosyltransferase (UFGTs) are mainly *O*-glycosyltransferases. There is considerable information regarding *O*-glycosylation, but there is only limited information on the *C*-glycosides. *C*-glycosides are found primarily in grasses but can also be found in some dicots such as *Fagopyrum esculentum* (Kerscher & Franz 1987). While *C*-glycosylation involving a *C*-glucosyltransferase to produce ACG1 has been reported (Brazier-Hicks et al. 2009), there is no information on the *C*-galactosylation step in ACG2 biosynthesis. Moreover, the genetic regulation of these two glycosylation events in relation to F2H is also unknown.

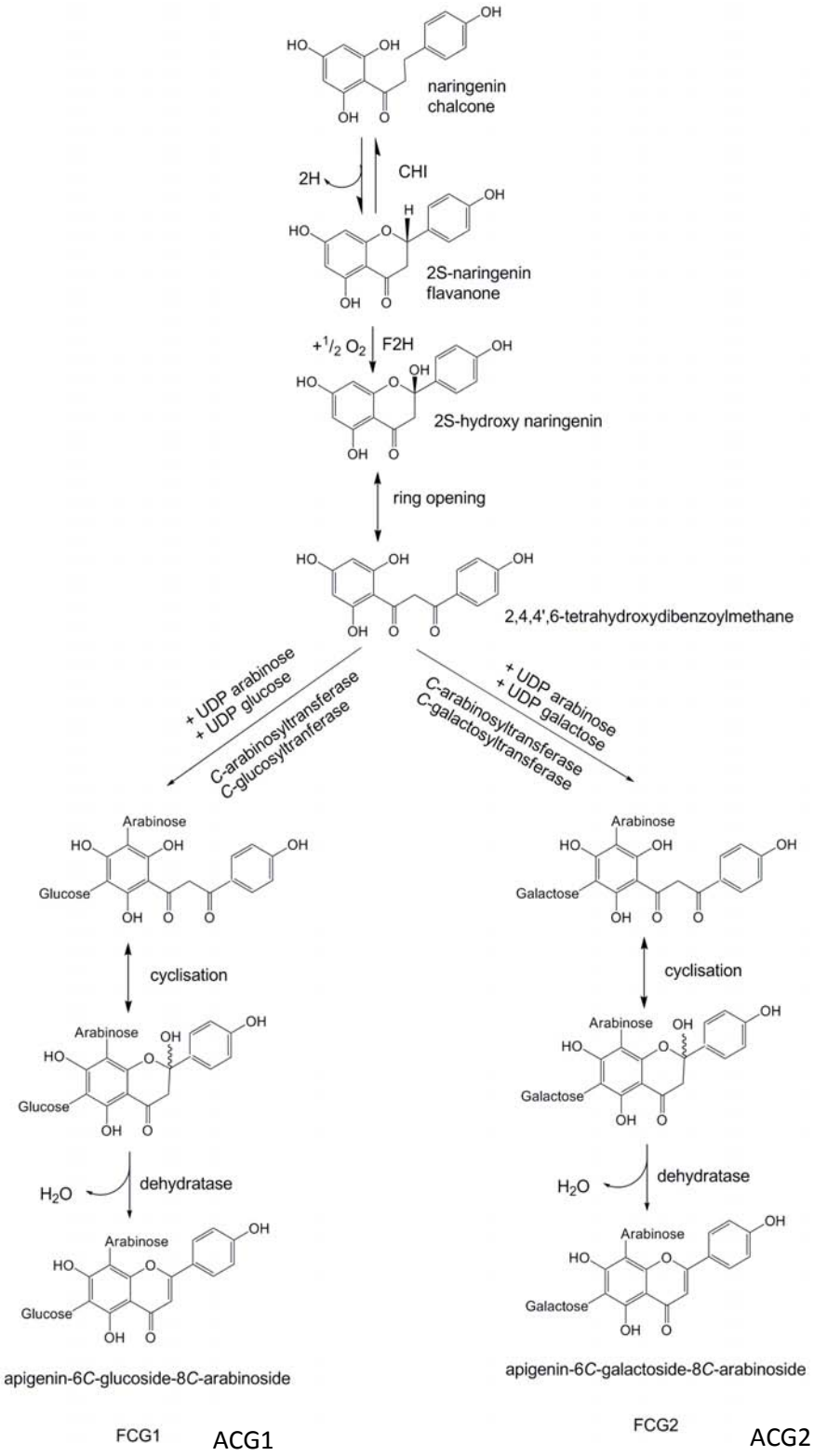

Figure 2.5 Proposed biosynthesis pathway for the 2 types of apigenin di-*C*-glycosides (ACG1 and ACG2) based on reports by Kerscher & Franz (1987), Brazier-Hicks et al. (2009) and Hamilton et al. (2009).

When this study began in 2008, information on *C*-glycosyltransferase genes and protein sequences was only available in the gene bank for actinomycetes (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein&cmd= search&term=c-glycosyltransferase; accessed 18th January 2009). They share 91% similarity with the plant *O*-glycosyltransferases, but their acceptor and donor substrates are different (Hoffmeister et al. 2002). However, the substrate-binding modes are highly conserved (Mittler et al. 2007). More recently, a *C*-glycosyltransferase gene and protein sequence has been reported in rice (Brazier-Hicks *et al.* 2009).

2.3.3 UDP-sugar recognition by the glycosyltransferases

Generally, glycosyltransferases are highly conserved in their secondary and tertiary structure (Osmani et al. 2009) and it appears unlikely that a single plant glycosyltransferase would have broad substrate recognition especially for the sugar donors (Kubo et al. 2004, Offen et al. 2006, Shao et al. 2005). However, Ono et al. (2010) reported evidence of a bifunctional UDP-glucose/UDP-galactose: flavonol-3-*O*-glucosyltransferase/galacosyltransferase activity in flavonol glycoside biosynthesis. Since the biosynthesis of the two ACG diasterioisomers is only differentiated by the hexose *C*-glycosylation pattern, the variation in contents of individual ACGs and subsequently the ratio may be an indication of genetic variation in the sugar donor specificity of the *C*-hexosyltransferases.

2.4 Genetic regulation of flavone biosynthesis in cereal grain

2.4.1 Genetic loci involved in the control of compounds related to the yellow colour of alkaline noodles

The b* values (yellowness) of noodles and the QTL/molecular markers associated with genetic variation have been mapped on chromosome arms 2DL, 2DS, 3AS, 3BL 4BS, 5BL, 7AL, and 7BL (Elouafi et al. 2001, Kuchel et al. 2006, Mares & Campbell 2001, Parker et al. 1998, Parker & Langridge 2000, Patil et al. 2008, Pozniak et al. 2007). QTL on the group 3 and 7 chromosomes are known to relate with xanthophyll biosynthesis (Mares & Campbell 2001, Parker et al. 1998). A microsatelite (SSR, simple sequence repeat) marker, *Xgwm344* on chromosome 7BL was linked to QTL for yellowness of noodles in durum wheat and bread wheat (Elouafi et al. 2001, Kuchel et al. 2006) and xanthophyll content (Harker et al. 2001). *Psy1-1,* a key gene involved in xanthophyll biosynthesis was mapped in the QTL region on chromosome 7A and 7B (Kuchel et al. 2006, Mares & Mrva 2001, Pozniak et al. 2007). Similarly, the QTL located on the group 3 chromosomes contains another key gene, Є-cyclase (*Є-LCY*), in the xanthophyll biosynthesis pathway (Howitt et al. 2009).

In a preliminary study, Mares and Wilsmore (unpublished data) identified a QTL on chromosome 7B using the map reported by Chalmers et al. (2001) that was associated with variation for the content of ACG1 and ACG2, the ratio of ACG1 and ACG2 and the total amount of both ACGs in grain in a Sunco x Tasman doubled haploid population. Statistical analysis showed a highly significant correlation between the ratio of ACG1/ACG2 and SSR marker *Xwmc76* that explained 83% of the variation in the population (Table 2.3, Figure 2.6). A fine map of this QTL needs to be generated to further explore these possibilities.

Table 2.3 DNA markers on chromosome 7B that were linked with ratio, ACG1/ACG2 in grain of Sunco x Tasman population (Mares and Wilsmore, unpublished data).

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Figure 2.6 Location of DNA markers on chromosome 7B of Sunco x Tasman (GrainGenes, accessed 22 September 2008).

2.4.2 Transcriptional regulation of flavonoid biosynthesis

Differential regulation of expression of sets of structural genes involved in different branches of the flavonoid metabolic pathway has been observed in a number of plants (Quattrocchio et al. 2006). However, functional redundancy was observed in the types of transcription factor proteins involved. Lepiniec et al. (2006) suggested that there was separate regulation of different sections of the pathway i.e. early biosynthetic genes

(EBG) and late biosynthetic genes (LBG) in *Arabidopsis thaliana*. Recent studies with different dicotyledonous plants has shown that the division into EBG and LBG varies for different tissues and plants (Deluc et al. 2008, Dubos et al. 2008, Gonzalez et al. 2008). In maize, all of the structural genes of the flavonoid metabolic pathway are induced simultaneously in coordination by several sets of transcription factors (Grotewold et al. 1998).

Similar communication and cooperation between transcription factors has been suggested in *Arabidopsis* and maize (Zhu 2006). Regulation by transcription factor interaction may be positive, resulting in the activation of structural genes and the production of certain group of compound at the end of the branch, or negative, resulting in the suppression of gene expression (Quattrocchio et al. 2006). The correlation between QTL or the gene for the expression of these transcription factor genes and the accumulation of structural gene transcripts and products have been previously studied in various plants including hexaploid wheat (Himi & Noda 2006, Khlestkina et al. 2008, Lagudah et al. 2001).

2.4.3 Transcription factors controlling flavonoid biosynthesis in cereal grain

There are two families of transcription factor proteins that are known to regulate the flavonoid biosynthetic pathway, R3R2-myb and bHLH. In *Arabidopsis*, interactions between these two families of transcription factors together with a WD40 repeat protein have been reported to lead to anthocyanin and proanthocyanidin biosynthesis, while the activity of R2R3-myb alone leads to the induction of flavonol and phlobaphene biosynthesis genes (Figure 2.7) (Quattrocchio et al. 2006). Slightly different regulation

is shown in maize where interaction between C1 and R, members of R2R3-myb and bHLH transcription factor respectively, only has been shown to result in the activation of anthocyanin, proanthocyanidin and flavonol glycoside biosynthetic genes (Goff et al. 1992). The activity of the myb transcription factor P alone results in the production of *C*-glycosyl flavones and phlobaphenes (Grotewold et al. 1998).

According to Himi et al. (2005 and 2011), *R* genes for grain coat colour code for *myb* transcription factors that upregulate *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase* (*F3H*), and *dihydroflavonol 4-reductase* (*DFR*). Whilst in red-grained wheats the *R* genes are associated with dormancy, it appears that expression of a dormant phenotype requires at least one other gene (Mares et al. 2005). Many red-grained wheat genotypes are non-dormant.

A study in rice suggested that the *R* locus in wheat might be orthologous with the *Rd* gene in rice (Sweeney *et al.* 2006). Both are located at homologous positions on chromosome 3 and 1 of wheat and rice respectively. However, the *Rd* locus of rice does not have phenotype when it is present alone (Sweeney et al. 2006) and encodes *DFR* to produce red seed colour in the presence of *Rc* gene (Furukawa et al. 2006). Moreover, a recent study by Balyan et al. (2008) argued that *Rd* of Rice was not orthologous with the *R* gene of wheat. The wheat genomic region containing the *R* gene appeared to be approximately 16Mb distal to the region containing the wheat homologue of the *Rd* locus of rice.

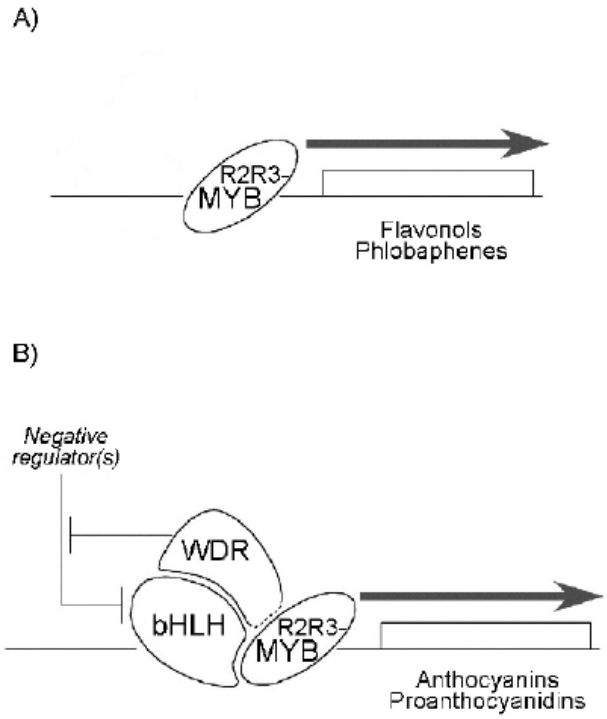

Figure 2.7 Activation of structural genes of flavonoid biosynthesis by transcription factors (Quattrocchio et al. 2006). A) The activity of R2R3-myb alone leads to the induction of flavonol and phlobaphene biosynthesis genes. B) Interaction between R2R3-myb, bHLH, and WD40 repeats (WDR) leads to anthocyanin and proanthocyanin biosynthesis.

The *Rc* gene, a *bHLH* transcription factor gene of proanthocyanidin biosynthesis on chromosome 7 of rice, encodes brown pericarp and seed coat when it is present without *Rd* (Sweeney et al. 2006). The study in wheat reported that the *Rc* loci for red pigmentation in coleoptiles is located on chromosomes 7A, 7B, and 7D (Khlestkina et al. 2002). They function as myb transcription factors for the expression of *F3H*, *DFR*, *ANS* (anthocyanidin synthase), and *UFGT* (*UDPG flavonol 3-O-glucosyl transferase*)

genes of flavonoid pathway that lead to anthocyanin biosynthesis (Ahmed et al. 2006, Himi et al. 2005, Khlestkina et al. 2008).

The *R* and *Rc* loci may regulate flavonoid metabolic pathway genes leading to anthocyanin biosynthesis in wheat similar to *Arabidopsis thaliana*, i.e. the *R* gene might be the transcription factor for genes in the early part of metabolic pathway (EBG) while *Rc* seems to be the transcription factor for the LBG. Other transcription factors such as *bHLH* and *WDR40* might also interact with the myb transcription factor in regulating FCG biosynthesis. However, it perhaps more likely that the regulation of *ACG* biosynthesis in wheat is similar to that of maize, where a myb transcription factor coordinates the flavone biosynthesis gene expression. In this model, the *R* gene or other myb transcription factor genes might coordinately control the expression of flavone biosynthesis genes in wheat from EBG to the LBG.

2.5 Wheat genomics for studying genetic regulation of flavone-di-*C*-glycoside biosynthesis

2.5.1 Organization of the wheat genome and wheat genomic information

The wheat species, *Triticum aestivum* L., commonly used for yellow alkaline noodles has the largest genome of all crop species, over 16,000 megabases (Gill et al. 2004) with a complex genome (Langridge et al. 2001). It is an allohexaploid species with 3 different genomes (A, B, D) originating from different ancestors (Lupton 1987). Within each genome, the genetic information is packed into 7 pairs of chromosomes (2n = 6x = 42) (Francki & Appels 2002). The genes in bread wheat are unevenly distributed along the chromosome interspersed with repetitive non-coding sequences (Flavell & Smith 1976). Approximately, 80% of the genome is occupied by repetitive (non-coding)

sequences and the rest consists of low copy number genes (Francki & Appels 2002). Akhunov et al. (2003) suggested that the genes are clustered in gene-rich islands that are dispersed throughout the chromosome length. Genome rearrangements and deletions that occurred during the species evolution without major detrimental effect on the plant have added greater complexity to the genome and therefore its study (Devos & Gale 2000, Langridge et al. 2001, Salina et al. 2006). Some genomic information (listed in Table 2.4) is available publicly online and can be useful for comparative genomic studies of plants.

Many studies have been performed on the organization of wheat genomes. Recently, the genomes of wheat have been fully sequenced by whole genome shotgun sequencing method (Brenchley et al. 2012) and the sequences have been made publicly available (www.cerealdb.uk.net). However, the sequences have not yet been fully annotated and assembled into chromosomes.

Table 2.4 Online Resources for comparative genomic study of plants.

Resource	Website	References
Gramene	http://www.gramene.org	Liang et al. (2008), Youens-Clark et al. (2010)
GrainGenes	http://wheat.pw.usda.gov/GG2/index.shtml	Carollo et al. (2005, Matthews et al. (2003a)
HarvEST	http://harvest.ucr.edu/	copyrights (C) 2001 - 2011 Steve Wanamaker, Timothy Close and the University of California
Model Crop	www.modelcrop.org	The International Brachypodium Initiative (2010)
ITEC	http://avena.pw.usda.gov/genome/	Lazo et al. (2004)
Triticeae EST-SSR Coordination	http://wheat.pw.usda.gov/ITMI/EST-SSR/	Matthews et al. (2003a)
National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/	National Center for Biotechnology Information (2005)
Maize Genetics and Genomics Database	http://www.maizegdb.org/	Dong et al. (2005)
Rice Genome Research Program	http://rgp.dna.affrc.go.jp/E/index.html	International Rice Genome Sequencing Project (2005)
TAIR	http://www.arabidopsis.org/	Garcia-Hernandez et al. (2002), Huala et al. (2001), Lamesch et al. (2012), Rhee et al. (2003), Swarbreck et al. (2008)
TIGR	http://www.tigr.org/	Ouyang et al. (2006)
DFCI Wheat Gene Index	http://compbio.dfci.harvard.edu	Quackenbush et al. (2001), Quackenbush et al. (2000)
TriAnnot Pipeline	http://urgi.versailles.inra.fr/projects/TriAnnot/	Michael (2012)
cerealsdb.uk.net	http://www.cerealsdb.uk.net/wheat.htm	Matthews et al. (2003b), Brenchley et al. (2012)
Catalogue of Gene Symbols for Wheat	http://wheat.pw.usda.gov/GG2/Triticum/wgc/2008/	McIntosh et al. (2008)
Komugi	http://www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp	Kurata & Yamazaki (2006)
Wheat Genome Info	http://www.wheatgenome.info	Berkman et al. (2011), Berkman et al. (2012)

2.5.2 Methods for studying regulation of gene expression in wheat

2.5.2.1 Reverse and forward genetic approaches for studying regulation of gene expression in wheat

Two approaches are widely used in studying gene function in plants, forward and reverse genetics (Peters et al. 2003). In forward genetics, the study of genetic regulation involves first observing the phenotype followed by identification of genes that are responsible for the traits (Alonso & Ecker 2006). In contrast, in reverse genetics, the study involves identifying gene sequences in a database followed by induced mutation or silencing of the gene and subsequently observation of the resulting phenotype.

The large size of wheat genome, high incident of repetitive non-coding regions and the existence of three genomes of the wheat discourage the application of reverse genetics in wheat (Stein & Graner 2004). As the gene sequences in plant species are generally conserved (Lagudah et al. 2001), the function of sequences from model plants or other cereals can be applied in wheat (Marshall et al. 2001). However, the homology and synteny between wheat and other cereal or model plant sequences is often only useful as a guide. The gene sequences and their order within the genome between wheat and other cereals may be very similar, but other features of the genome such as regions between genes and the non-coding sequences can be significantly different (Francki & Appels 2002). The occurrence of numerous translocation, inversion, insertion and deletion events during evolution may also hinder the discovery of homologous gene sequences (Francki & Appels 2002, Gill et al. 2004, Lagudah et al. 2001). Depending on the level of genetic relatedness, comparison of genomes should also take into account the similarity in gene products and physiological features associated with their growth and development (Lagudah et al. 2001). Deducing the gene homology through

similarity of gene products might be more suitable in comparing wheat and model plant genomes than direct gene sequence alignment (Lagudah et al. 2001).

Unfortunately the application of reverse genetics in wheat is not always straight forward and several important molecular techniques such as southern blot and genetic transformation are difficult to apply, with significantly higher rates of failure in wheat than in other plants (Langridge et al. 2001). The presence of the three different genomes of wheat means three sets of bands will usually appear in many molecular marker analyses particularly restriction fragment length polymorphism (RFLP). The identification of gene sequences in wheat mainly depends on the map based cloning approach of forward genetics (Francki & Appels 2002). Initial work to identify wheat transcription factor genes are mainly performed by fine mapping, especially when the previous sequence information is unavailable (Stein & Graner 2004). Transcript analysis can be performed following the fine mapping by reverse transcription polymerase chain reactions (RT-PCR) based techniques (Ahmed et al. 2006, Himi et al. 2005, Himi & Noda 2006) after the candidate gene sequences are successfully identified.

2.5.2.2 Identification of transcription factor genes through genetic mapping and positional cloning

Genetic mapping and positional cloning have been used to isolate genes from complex genomes. Co-localization of genes on genetic and physical maps is used to identify DNA sequences by aligning the physical and genetic maps and placing the genetic markers on the physical map and the DNA fragments on the genetic map to validate both maps (Goyal et al. 2005, Liu 1998). The order of the linked genes in both maps is

usually conserved and the genes can be ordered precisely on the physical map (Stein & Graner 2004).

A wide range of resources are available for genetic mapping. Nullisomic-tetrasomic series as well as ditelosomic lines of wheat variety Chinese Spring are available to locate genes controlling agronomically important traits to specific chromosome or chromosome arms respectively (Lupton 1987). Moreover, the availability of molecular markers that have been placed into either genetic or physical maps of various wheat breeding populations make it possible to perform linkage analysis at high resolution (fine mapping) (Langridge et al. 2001). Good levels of polymorphism have been shown in the application of SSR markers within Australian germplasm (Chalmers et al. 2001, Harker et al. 2001, Parker et al. 1998) although polymorphisms are more difficult to identify in wheat than in other species (Langridge *et al.* 2001). In a study by Harker et al. (2001), around 148 polymorphic SSR markers were identified in a Sunco × Tasman doubled haploid population with 93% loci located in linkage groups. Chalmers et al. (2001) reported 11 polymorphic DNA markers, but few SSR markers, in the region of chromosome 7B in Sunco x Tasman where the QTL for ACG1/ACG2 ratio of ACGs is located (Mares and Willsmore, Unpublished). However, more recent publications involving other wheat populations suggest that there are many SSR markers on the short arm of 7B chromosome that could be tested (Ganal & Röder 2007). Information on the various aneuploid and chromosomal mutant lines of wheat that can be used for assigning SSR markers to linkage groups and for physical mapping is available on the GrainGenes website (Langridge et al. 2001).

Some loci on 7BS were observed to be homologous to those on 5BL and 5DL instead of 7AS and 7DS (Nelson et al. 1995) and may be useful for saturating the QTL region on 7BS. The translocation, inversion and re-arrangements between 4A, 5A, and 7B chromosomes during the polyploidization in hexaploid wheat evolution impacted on the recombination in these chromosomes (Devos et al. 1995, Salina et al. 2006). The genetic map and physical map of group 7 chromosomes of hexaploid wheat was reported to be collinear (Hohmann et al. 1994), as many as 54 homologous loci have been located on chromosomes 7A, 7B, and 7D with almost identical linear order of the molecular markers between the 3 chromosomes. However, the physical distant was not correlated with the genetic distant and some distortion in distal regions of chromosomes were identified.

The identification of QTL may require the development of a new population from parents with more extreme phenotype, but for which a map is not available. Diversity arrays technology (DArT) that types hundreds to thousands of genomic loci in parallel can be applied to provide such a map (Semagn et al. 2006). For routine genotyping of hexaploid wheat, the maps generated by this technology provide preliminary genome coverage with information and quality comparable to combined SSR/RFLP/AFLP (amplified fragment length polymorphism) maps (Akbari et al. 2006).

2.5.2.3 Availability of resources for synteny analysis to determine transcription factor gene sequences

Whilst a fully annotated wheat genome is not yet available , resources such as the BAC library and EST sequence database of wheat may be used to construct DNA sequences containing the genes of interest (Gill et al. 2004, Moolhuijzen et al. 2007). The

information on the macrosatellite and SNP marker based deletion bin system for establishing relastionships between physical and genetic maps are also publicly available (Akhunov et al. 2010, Sourdille et al. 2004b). The availability of full sequence information of syntenic chromosomes in rice and *Brachypodium distachyon* assisted in the organisation of the contigs and establishing the relationship between the physical map and the fine genetic map of a chromosome. In this way, the physical map of group 3 chromosome of wheat and the respective genetic map has been developed (Dilbirligi et al. 2006, Fleury et al. 2010, Paux et al. 2008, Stein 2007).

So far, the BAC library of bread wheat is available mainly for the A and D genomes, (http://wheatdb.ucdavis.edu:8080/wheatdb/) (Lijavetzky et al. 1999, Shen et al. 2005, Stein 2007) that were sequenced from the diploid species *Triticum uratu* and *Aegilops tauschii*. The relationship between these diploid genomes and the hexaploid bread wheat genomes have also been established through the genetic maps (Boyko et al. 2002, Gill 1991). For the B genome, a BAC library of the S-genome of *Aegilops speltoides* that has been reported to be closely related to the B genome of hexaploid bread wheat, is also available (Akhunov et al. 2005). In addition, the BAC library for the B genome is also available for the tetraploid wheat relative, *Triticum turgidum* spp. durum (Cenci et al. 2003). This BAC library should also be useful for a comparative genomic study, as the B-genome chromosomes of this species and bread wheat are >99% identical (Kubalakova et al. 2005).

The wheat EST database contains the most current wheat genomic information available. In 2004, more than 500,000 sequences were stored in the wheat database, representing around 60% of the wheat genes (Gill et al. 2004). In 2009, this had

increased to more than 1,000,000 sequences for wheat and closely related species stored in various EST databases (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html; verified 6 Jan 2009; http://www.plexdb.org/). Furthermore, thousands of ESTs have been mapped to wheat genetic and physical maps as single genes and used to reveal wheat-rice genome colinearity (Adom et al. 2003, Stein 2007). In addition, database and other resources for mapping the EST sequences into the deletion bin map have been developed (Lazo et al. 2004). A chromosome bin map for group 7 homologous chromosomes with 2148 EST was reported (Hossain et al. 2004). Similar information was also reported for 1918 EST loci in wheat homologous chromosome 4.

Besides the wheat EST database, the complete genome sequence of rice is also available for analysing synteny (Salse et al. 2008). DNA marker distributions along physical chromosome maps of these two cereals are generally collinear (Dunford et al. 1995). The distance may be different, but the order of the markers and genome structure are generally conserved (Kurata et al. 1994, Moore 1995). From the distribution of EST sequences in physical maps of wheat deletion lines and their segregating populations, group 7 wheat chromosomes are known to be collinear with a combination of several segments of rice chromosomes. The short arms of wheat chromosome 7 are matched with short arms of rice chromosome 6 and long arms of chromosome 8 while the long arms of wheat chromosome 7 are matched with the long arms of rice chromosome 6 and short arms of chromosome 8 (Rota & Sorrells 2004).

In addition to the rice full genome sequence, other sources for genome comparison are also available. The genome of a new model plant for temperate grasses and cereals, *Brachypodium*, should be useful in bridging the gaps between wheat and rice (Hasterok

et al. 2006). The BAC library of this species has been available for synteny analysis since early 2009. Moreover, a BAC „landing" method for comparative genome alignment between cool season grasses such as wheat and *Brachypodium* has also been published (Jenkins & Hasterok 2007).

The assembly of gene sequences by genome comparison and sequence alignment results in putative sequences of interest (or contigs) (Gupta et al. 2008). Unfortunately, ESTs for genes with low expression might not be detected whilst transposable elements and alternatively spliced gene sequences might be misidentified as multiple unigenes (Stein 2007). In addition, small translocations, deletions, inversions, and duplications often violate colinearity and complicate the synteny analysis (Gill et al. 2004). After the putative gene sequences are obtained, confirmation of their function is performed through gene transcript analysis (Leader 2005).

2.5.2.4 Transcript analysis of wheat genes

In previous studies (Ahmed et al. 2006, Himi et al. 2005, Himi & Noda 2004, Khlestkina et al. 2008), the expression profiles of transcription factor genes have been shown to be correlated to the expression of biosynthetic genes. The transcript analysis is performed by Reverse Transcript PCR techniques. When the information on the structural gene sequences is unavailable for the plant under study, a bioinformatics analysis is first performed to identify these sequences in the EST library by using available sequences as references. This can be done before correlating the expression level of the transcription factor loci resulting from fine mapping to the structural gene transcripts and to the metabolic content (Lagudah et al. 2001).

2.6 Conclusion

As the yellow colour of yellow alkaline noodles (YAN) influences their market value, improvement of the natural yellow colour compounds in wheat grain and flour is important. Apigenin di-*C*-glycosides (ACGs) in wheat grain make a significant contribution to the yellow colour YAN. It is therefore desirable to improve the ACG content in wheat grain, but unfortunately there is only limited information available on the genetic variation for ACG content in Australian or other wheat cultivars. Similarly, whether existing populations are suitable for further genetic mapping study remains to be determined and there is no information on the enzymes and the genes involved in the critical part of flavonoid pathway that leads to ACG biosynthesis in wheat grain. However, there is information on the enzymes that code for flavone biosynthesis in wheat and related plants and transcriptional regulation of the general flavonoid pathway that can be used as a starting point for studying biosynthesis of ACG.

QTL and SSR markers linked to variation in ACG composition in a specific wheat population (Sunco x Tasman) have been identified. Fine mapping of this locus and a comparative genomic study with the aid of EST and BAC libraries available for wheat and other cereals are required to identify the candidate gene sequences involved in ACG biosynthesis. These sequences could be the structural gene or a transcription factor gene(s) involved in ACG biosynthesis. A correlation between the expression of these candidate sequences and ACG synthesis during grain development would verify their importance to the ACG pathway.

Potentially, there are myb, bHLH and WDR40 transcription factors that regulate other branches of the flavonoid pathway, could regulate ACG biosynthesis as well. The *R*

genes that encode myb transcription factors in wheat and orthologues in other cereals including rice and maize that are important in regulating large portions of the flavonoid pathway might also be involved in regulating ACG synthesis in wheat grain. A sequence analysis followed by transcript analysis to correlate the expression of these transcription factor genes with the structural gene transcripts, and the ACG concentration would give information regarding their importance to ACG biosynthesis.

2.7 Research questions

The overall aims of this project are to quantify the contribution of apigenin di-*C*-glycosides to the colour of YAN and to investigate the genetic regulation of their biosynthesis in wheat grain.

2.8 Research aims

The specific aims of this project will be:

- to survey the genetic variation in grain ACG content and composition within Australian bread wheat cultivars.
- to determine the quantitative role of ACG in the yellow colour of YAN
- to locate and to validate the QTL associated with ACG content, concentration and composition in bread wheat grain.
- to fine map the highly significant QTL region on 7BS.
- Survey genes likely to be located in the fine-mapped region on chromosome 7B for candidate genes involved in determining ACG composition

2.9 Significance/contribution to the discipline

This research will provide new information on the role of apigenin di-*C*-glycosides in the colour of YAN and the genetic control of the biosynthesis of these compounds in wheat grain. Moreover, the research may also show whether all the genes involved in flavone biosynthesis in wheat grain are co-ordinately regulated by a set of transcription factors, or whether there are separate regulators for early and later stages of the pathway.

Chapter III: General Materials and Methods

3.1 Introduction

In this chapter, the background on the materials and methods that have been used in this thesis are outlined and explained. The details on the use of these materials and methods for specific work have been described in the relevant chapter.

3.2 Plant Materials

3.2.1 Genetic resources for experiment 1 (Chapter IV): Survey of genetic variation in ACG content and composition

Seventy bread wheat (*Triticum aestivum* L.) genotypes were sourced primarily from Australian breeding programs, but also included a small number of varieties of miscellaneous origin. The complete list of these cultivars is in supplemental material 4.1. Forty four of these cultivars were released by various Australian breeding program in 5 different states Queensland, New South Wales, Victoria, South Australia and Western Australia. Twenty six of the cultivars from miscellaneous sources were obtained either from The University of Adelaide pre-breeding program (10 cultivars), 2 from Leslie Research Center in Queensland, 2 from South Africa, 5 from China, 1 from Japan whilst 2 cultivars and 4 synthetic derived hexaploid wheats originated from Mexico. In 2009, 29 genotypes from the Australian breeding program that cover the range in ACG content were selected for a G X E experiment.

The diploid, tetraploid, and hexaploid relatives of breadwheat: accessions of *Triticum monococcum (*A^mA^m*)*, *Triticum uratu (*AA*)*, *Aegilops tauschii* (DD)*, Aegilops speltoides* (SS)*, Triticum dicoccum* (AABB)*, Triticum polonicum* (AABB)*, Triticum*

turgidum (AABB), *Triticum diccoides* (AABB), *Triticum carthlicum* (AABB), *Triticum durum* (AABB), *and Triticum spelta* (AABBDD) were obtained from the Australian Winter Cereals Collection, Tamworth; while 5 *Triticum durum* cultivars were obtained from Australian breeding program in NSW (4) and South Australia (1) (supplemental material 4.1).

Aneuploid lines: The full set of 42 nullisomic-tetrasomic lines of Chinese Spring (Sears 1981) were obtained from Ms. Margie Pallotta of the Australian Centre for Plant Functional Genomics, The University of Adelaide. A set of Chinese Spring/*Triticum spelta* chromosome substitution lines (21 lines where each Chinese Spring chromosome in turn was substituted with the homologous *T. spelta* chromosome) was sourced from the John Innes Research Centre, UK.

3.2.2 Genetic resources for experiment 2 (Chapter V): Quantitative assessment of content and composition of the ACGs and their contribution to YAN colour in comparison to that of lutein

Twenty eight cultivars of Australian commercial bread wheat (*Triticum aestivum* L.) representing all the wheat production zones in Australia, that were grown in a field trial at Turretfield in South Australia in 2005, were used both for analyses of grain ACG content and composition as well as for preparation of flour using a Quadrumat Junior Mill. Ten were re-sown in 2009 to provide sufficient sample size for Bühler milling.

Sunvale (Australian Premium Hard quality in NSW and Queensland region and Australian Hard quality in other states including South Australia) flour was used as the base for construction of a standard curve quantifying the effect of addition of different

amounts of rutin on YAN colour. Suneca (Australian Premium Hard quality in NSW and Queensland region and Australian General Purpose quality in other states including South Australia) was used as a control to monitor the milling process.

3.2.3 Population for experiment 3 (Chapter VI): Genetic control of the ACG content and composition in bread wheat grain

3.2.3.1 Parents of Australian doubled haploid populations

Initially, parents of 7 existing bread wheat doubled haploid populations, Sunco/Tasman, Sunco/Aroona, Avocet/Sunvale, Avocet/Angus, Avocet/Sunfield, Sunvale/Sunfield, and Cranbook/Halberd were grown in Turretfield, South Australia in 2005. Their grains were analysed and compared for ACG content and composition.v

Of these, Sunco/Tasman was chosen for further study since the parents were shown to vary significantly for ACG composition, although not significantly for total ACG content, (Supplemental materials 3.1, 3.2, and 3.3) and because a genetic map was already available (Kammholz et al. 2001). This doubled haploid population has also been widely used for mapping other traits related to noodle colour (Mares and Campbell, 2001).

3.2.3.2 Sunco/Tasman doubled haploid population used in the QTL mapping

Whilst there were 269 doubled haploid lines generated from the cross, Sunco/Tasman, the lines used as the mapping population represent the first 180 transferred from culture (Kammholz et al. 2001). Due to high segregation distortion in the molecular marker data (Chalmers et al. 2001, Kammholz et al. 2001), this population has been sub-

sampled in the further studies examining the segregation of various major genes. In 2008, the genotype (308 markers) and phenotype data used in these studies were made available in MapManager database. These data were provided by Dr. Anke Lehmensiek and Dr. Kerrie Willsmore, and converted into excel spread sheet by Dr. Greg Lott. As many as 203 of the original Sunco/Tasman doubled haploid population were phenotyped for ACG content, ACG composition, 100 grain weight, ACG1 content, ACG2 content, total ACG per gram and total ACG per grain in 2005 by Mares and Asenstorfer (unpublished data). Only 147 lines from these 2 data sets had matching identification. In 2010, another set of genotyping data of 178 lines with 361 markers was obtained from Professor Rudi Appels, Murdoch University, and Prof. Mrinal Bhave and Dr. Huimei Wu (Swinburne University of Technology). Markers on chromosome 1A and 7B were used to compare this data set with the 2008 genotyping data (Lehmensiek, Willsmore and Lott). A new set of Sunco x Tasman doubled haploid population data was compiled with 153 lines and 361 markers, to validate the preliminary QTL mapping of ACG traits performed by Mares and Willsmore (Unpublished data).

In 1999, seed of 271 doubled haploid lines, provided by Dr S. Kammholz, Leslie Research Centre, Toowoomba, Queensland, Australia, was increased in a field trial at Narrabri in New South Wales, Australia. Sunco and Tasman parent lines were planted alternately every 20th plot within the trial (Supplemental material 3.4A and 3.4B). The grains of these lines were stored at -20^0C until required for this project.

Four SSR markers: *wmc76*, *gwm400, gwm573* and *wmc364* on 7BS that flanked the QTL region for ACG1, ACG2, and ACG1/ACG2 ratio were used to compare this

population with the previous mapping data (Lehmensiek et. al in 2008, Bhave et. al in 2010, Mares and Asenstorfer unpublished data). The multiplex PCR results for the other markers, *gwm400* and *wmc76* were confirmed by the second PCR and gel electrophoresis visualisation. The result for *wmc364* was confirmed by the second data set from Bhave et. al in 2010.

The distribution of the new phenotypic data for the first set of population lines (163 lines) was compared with the additional lines of the population from as a second group (108 lines). Four lines of the final compilation of the genotypic and phenotypic data set did not have phenotypic data because the grain was unavailable. The frequencies of ACG1/ACG2 ratio data of these two groups were distributed with the first group have slightly higher ratio range than the second group (Supplemental materials 3.5). The low ratio data distributed similarly, but the high ratio data were slightly higher for the first group than the second group. The differences in the trait distribution may be a result of more than one F_1 used in generating the mapping population (Kammholz et al. 2001).

3.2.3.3 Other genetic stocks used in marker analysis

A panel of Sunco/Tasman doubled haploid lines were chosen for polymorphism test of the SSR markers. This panel consisted of 10 lines with ratio similar to Sunco (high ratio) and another 10 lines with ratio similar to Tasman (low ratio). Two pairs of lines of these population parents were also included in the test. The nullisomic-tetrasomic lines of Chinese Spring group 7 chromosomes (section 3.2.2) and ditelosomic lines group 7 were used to confirm the chromosome arm location and designing the SNP markers. The ditelosomic lines were obtained from Prof. R. Mc Intosh, University of Sydney, New South Wales, Australia.

3.3 Location, time, and condition of planting of the genetic resources

Locations and time of planting of the genetic resources used in this thesis is presented in Table 3.1. For those grown in the glasshouse, the seeds were germinated on filter paper in a petri disk moistened by 4 mL water and stored in the dark at room temperature for 3-4 days. The seedlings were then transplanted into coconut fibre potting mix and keep in the glasshouse with controlled environment. For those grown in the field, the seeds were directly sown in the soil.

Table 3.1 Locations and time of planting of the genetic resources used in this thesis.

Experiments	Plant Materials	Location of planting	Year
Survey of genetic variation	70 bread wheat cultivars	Field, Turretfield, South Australia in 2005	2005
	35 relative species of bread wheat	Glasshouse, Waite Campus of the University of Adelaide	2009
	Chromosome modification and substitution lines	Glasshouse, Waite Campus of the University of Adelaide	2009
	29 lines for G X E experiment	Field, Turretfield, South Australia in 2005 and Waite Campus of the University of Adelaide	2009
	F1 generations	Glasshouse, Waite Campus of the University of Adelaide	2009
Quantification of ACG contribution to YAN colour	28 cultivars for Udy cyclone Mill and Quadrumat Junior Milling	Field, Turretfield, South Australia in 2005	2005
	10 cultivars for Bühler milling	Field, Turretfield, South Australia in 2005	2009
QTL analysis and fine mapping	Sunco x Tasman doubled haploid population	Glasshouse, Plant Genomic Center, Waite Campus of the University of Adelaide	2009
	Nullisomic-tetrasomic lines and ditelosomic lines of group 7	Glasshouse, Waite Campus of the University of Adelaide	2009

3.4 Phenotyping

3.4.1 Data collection

3.4.1.1 ACG analysis

Methods for extraction and quantification of ACGs were as reported by Asenstorfer at al. (2007), Asenstorfer et al. (2006) and Ingram (2006) with minor modifications. Initially, the grain weights were recorded. The grains were ground to a fine meal using a 3 M ESPE Rotomix, and 0.1 g of the whole meal mixed with 1.8 mL of 0.1 M hydroxylamine (Sigma, St Louis, USA) pH 7.0. Subsequently, each sample was spiked with 50 μL of vanillin standard (Sigma, St. Louis, USA) and mixed for 24 hours prior to centrifugation at 2100 g for 10 minutes. The supernatant was then loaded onto a reverse phase SPE (Strata^{TM} -X33μm C18 polymorphic sorbent, Phenomenex, Torrance, CA, USA) column preconditioned with methanol and water. ACGs were eluted from the column using methanol (technical grade, Crown Scientific) the eluate spiked with 50 μL rutin (Sigma, St. Louis. USA) and then vacuum concentrated. The residue was re-suspended in 250 μL of 50% methanol.

The HPLC analysis was performed on a Hewlett-Packard HPLC 1100 instrument equipped with an autosampler, column heater and diode array detector. Separation was achieved on a C18 column (Platinum EPS 100A 3 μ 53x7 mm; Alltech, Illinois, USA). Two solvents, A (HCOOH (Merck KGaA, Darmstaat, Germany)/H_2O (1:99 v/v)) and B (HCOOH (Merck KGaA, Darmstaat, Germany)/CH_3CN (Gradient 240nm/far uv, HPLC grade, Scharlau, Sentmenat, Spain)/MeOH (Isocratic HPLC grade, Scharlau, Sentmenat, Spain) (1:4:95 v/v/v) were used as eluents, with elution at a flow rate of 0.65 mL/min according to the following elution profile: 0-3 min, isocratic B; 3-8 min gradient 10%B to 24% B; 8-11 min, isocratic 24% B; 11-18 min, gradient 24% B to 34% B; 18-28 min,

gradient 34% B to 44% B; 28-35 min gradient 44% B to 65% B; 35-40 min, gradient 65% B to 95% B; 40-55 min, isocratic 95% B; 55-60 min, gradient 95% B to 10% B; 60-70 min isocratic 10% B. Analytical wavelengths, UV 340 nm, was used for detection and quantification of the ACG.

This method was used in all ACG analysis in this study, except for analysis of ACG content in flour, which will be explained in the relevant chapter (Chapter V).

3.4.1.2 The Use of Internal and external standard solutions in ACG Analyses

3.4.1.2.1 The use of vanillin internal standard in ACG analyses

Vanillin was used because it is not present in wheat grain, it elutes in a region of the chromatogram that is free of other constituents, and is stable under the sample preparation and HPLC conditions used in this study. Vanillin was added after extractant and sample were mixed and used primarily to monitor any losses during the reverse phase SPE step and extract drying. The recovery was consistent throughout and very similar to the recovery of pure vanillin on its own. In rare cases where it was not, the samples were repeated.

Two replicates of controls contained blank samples (without whole meal materials) were spiked with 50 μL pure vanillin standards (2.5 mg/mL stock solution, Sigma, St. Louis, USA). The analytical 280nm UV wavelength detected the peak of vanillin standard at 8.322 minutes. The areas of the vanillin peaks obtained from these controls and from the samples were then compared and calculated as in section 3.4.2.1 to get the vanillin corrected area values.

3.4.1.2.2 The used of rutin internal standard

Rutin (50 μL, from 0.5mg/mL stock solution, Sigma, St. Louis. USA) were used to spike the ACG samples eluted from the SPE column using methanol prior vacuum concentrating. Two controls of pure rutin standard at similar concentrations were also prepared. The analytical 340nm UV wavelength detected the peak of rutin standard in these samples and controls at 16.492 minutes. The areas of the rutin peaks obtained from every sample and from each sets of HPLC measurments were compared. In rare cases where differences were occured, the samples were repeated, and if the differences stayed, the rutin area values were used to correct the differences between these sets of measurements.

3.4.1.2.3 The use of rutin external standard

A series of rutin dilutions (10 to 1000 times) were prepared from 1 mg/mL of rutin stock solution in 50% methanol (isocratic HPLC grade, Scharlau, Sentmenat, Spain). The areas of these rutin dilutions were measured by HPLC at 340nm UV wavelength. To construct the standard curve, these areas were plotted as abscissa againts concentrations of rutin as ordinate in Microsoft Excell worksheet (version 2007). A trendline and the coefficient of this standard curve were regenerated to calculate the rutin equivalent of ACG content in the samples as in section 3.4.2.1.

3.4.1.3 Hundred grain weight

The 100 Grains were counted using a seed counter (CONTADOR, Pfeuffer GmbH) and the weight was measured using an analytical balance.

3.4.2 Data Analysis

3.4.2.1 Calculations of ACG traits

Seven ACG content and composition traits were calculated from the raw data of ACG1 and ACG2 obtained from HPLC analysis: concentration and content per grain for ACG1, ACG2 and total content of and ACG1/ACG2 ratio.

Firstly, the vanillin corrected areas were calculated as follows:

$$\frac{\text{area of pure vanilin std}}{\text{area of vanilin std in each samples}} \times \text{Area of ACG1 or ACG2}$$

then, the volume corrected area were calculated as follows:

$$\text{Vanilin corrected area (for each ACG1 or ACG2)} \times \frac{\text{final dilution volume (µL)}}{1000}$$

The concentrations of each ACG1 and ACG2 content per gram sample (µg/gram) were calculated as follows:

$$\frac{\text{Volume corrected area}}{\text{standard curves coefficient}} \times \frac{1000}{\text{sample weight (g)}}.$$

while each ACG1 and ACG2 content in a grain (µg/grain) were calculated as follows:

$$\text{Concentration of ACG1 (or ACG2) in µg/g sample} \times \text{weight (g) per 1 grain}$$

The total content of ACGs was calculated as:

$$\text{ACG1 content} + \text{ACG2 content}$$

and the ACG1/ACG2 ratio were calculated as:

$$\frac{\text{ACG1 content}}{\text{ACG2 content}}$$

3.4.2.2 Statistical Analysis

All statistical analyses were performed in GENSTAT software (version 14; VSN International, Hemel Hempstead, UK). Details of these analyses were explained in relevant chapters.

Title of Thesis: Genetic control of Apigenin di-*C*-glycosides biosynthesis in bread wheat (*Triticum aestivum* L.) grain and their potential as yellow pigment of Asian alkaline noodles

Student Name: Grace Yasmein Wijaya

Pg # PhD

CHAPTER IV

Chapter IV: Apigenin di-C-glycosides (ACG) content and composition in grains of bread wheat (*Triticum aestivum*) and related species

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Pg # PhD

STATEMENT OF AUTHORSHIP

Title of Paper Apigenin di-C-glycosides (ACG) content and composition in grains of bread wheat (*Triticum aestivum*) and related species

Journal of Cereal Science ; 2012: accepted paper

Name of candidate Grace Yasmein Wijaya

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Designed and developed the detail experiment for data collection, performed the biochemistry analysis on all samples (excluded HPLC machine set-up), analyzed and interpreted the data, planned the article, wrote manuscript and acted as corresponding author

Certification that the statement of contribution is accurate

Signed ... .*Date*..... 19 Maret 2012

co-author name Assoc. Prof. Daryl Mares

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Principal supervisor of the project, developed the initial outline for the project, provided background IP for the project, all the germplasm and genetic stocks required, provided critical evaluation, supervised development of work and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed ....*Date*..... 20/3/2012

Chapter IV: Apigenin di-C-glycosides (ACG) content and composition in grains of bread wheat (*Triticum aestivum* L.) and related species

4.1 Introduction

Apigenin di-*C*-glycosides (ACGs) are present in the grain of bread wheat and other related cereals primarily as one or two sets of Wesseley-Moser isomers containing either arabinose and glucose (ACG1) or arabinose and galactose (ACG2) on the A ring of apigenin. These compounds may contribute to the yellow colour of wheat-based products made under alkaline conditions and in addition, have possible roles in a number of plant physiological processes and human health. However, despite their diverse and important roles, the biosynthesis of these compounds in plants is still not well understood.

In wheat (*Triticum aestivum* L.) grain, Asenstorfer et al. (2006) reported significant variation in the content of ACG1 and ACG2 and the ratio ACG/ACG2 for a small set of Australian wheat cultivars. It is unclear whether the differences in ACG1 and ACG2 content, which is reflected in the ratio, are the result of two different *C*-hexosyltransferases, *C*-glucosyltransferase and *C*-galactosyltransferase, or a single *C*-hexosyltransferase that recognizes both uridine 5′-diphosphoglucose (UDP-glucose) and uridine 5′-diphosphogalactose (UDP-galactose).

The aims of this investigation were to survey genetic variation for ACG content and composition in hexaploid bread wheat (*Triticum aestivum* L.) and to examine ACGs in

the putative progenitors of hexaploid wheat, F_1 grains derived by crossing bread wheat parents with different ACG phenotype, Chinese Spring nullisomic-tetrasomic lines and chromosome substitution stocks as a first step towards understanding the mechanisms involved in their biosynthesis and genetic control.

4.2 Material and Methods

4.2.1 Plant materials

Seventy bread wheat (*Triticum aestivum* L.) genotypes sourced primarily from Australian breeding programs, but including a small number of varieties of miscellaneous origin, were grown as single twin row plots, 4 m long, at Turretfield, South Australia in 2005. The harvested grains were analysed for ACG content and composition in order to survey variation for these traits. Genotypes surveyed were as follows (Supplemental material 4.1):

a) Cultivars released by Australian breeding programs – Batavia, Baxter, Cook, Cunningham, EGA Gregory, EGA Hume, Hartog, Janz, Kennedy, Lang, Spica, Tasman (Queensland); Gabo, Gamut, Gatcher, Sunco, Suneca, Sunfield, Sunlin, Sunvale, Ventura (northern New South Wales); Avocet, Diamondbird (southern New South Wales); Annuello, Chara, Meering, Silverstar (Victoria); Angus, Aroona, Frame, Halberd, Krichauff, Kukri, Spear (South Australia; Canna, Carnamah, Cascades, Cranbrook, GBA Ruby, Reeves, Westonia (Western Australia); b) Genotypes from the University of Adelaide pre-breeding program - DM03.122/WI21121.1151, DM03.122/WI21121.1442, DM03.24.b.241, DM03.24.b.248, DM03.24.b.281, DM5686*B12, Sunco/Indis.82, SW95-50213/Cunningham.763, SW95-50213/Cunningham.799, and c) Miscellaneous – AUS1408, Indis (Africa); Chinese Spring, ChuanYu12, ChuanMai18, Jing Hong (China); Haruhikari (Japan); Ciano, Seri,

synthetic derived hexaploid wheats AUS29565, AUS29572, AUS29604, AUS30330 (Mexico); Condor high molecular weight glutenin biotypes 17 and 19, SUN325B, Yellow Kite (University of Sydney); QT7475 (Leslie Research Centre, Queensland); AUS1490 (Australian Winter Cereals Collection, Tamworth, New South Wales).

In 2009, 29 genotypes selected from this set to cover the range in ACG content were sown in randomized replicated trials (twin row x 4 m plots) at 2 locations: Turretfield Agricultural Research Centre (TARC), South Australia (34.97 ^{o}S latitude and 138.63 ^{o}E longitude) and Waite Campus, University of Adelaide, Urrbrae, South Australia (34.55 ^{o}S latitude and 138.83 ^{o}E longitude). TARC is located on red-brown earth in the livestock-cereal zone 55 km north of Adelaide at 115m elevation with mean annual minimum and maximum temperatures of 12.2 ^{o}C and 22.3 ^{o}C respectively and 542.2 mm rainfall, of which 450.6 mm occurred during April-Dec 2009 (Australian Bureau of Meteorology, Climate Information, online data access: March 2nd, 2011). The Waite campus is located on the black cracking clay soil of the Adelaide plains at 116 m elevation with mean annual minimum and maximum temperatures of 10 ^{o}C and 22.5 ^{o}C respectively and 467.3 mm rainfall, of which 451.6 mm occurred during April-Dec 2009. The results from this field trial were required in order to gain a preliminary estimate of the effects of genotype (G), environment (E) and GxE on ACG content and composition.

In addition, accessions of *Triticum monococcum*, *Triticum uratu*, *Aegilops tauschii*, *Triticum durum*, *Triticum spelta*, *Aegilops speltoides*, *Triticum dicoccum*, *Triticum polonicum*, *Triticum turgidum*, *Triticum diccoides*, and *Triticum carthlicum* (section 3.2.1); the full set of 42 nullisomic-tetrasomic lines of Chinese Spring (section 3.2.1)

and the Chinese Spring/*Triticum spelta* chromosome substitution set (section 3.2.1) were all subsequently grown in a glasshouse at the Waite Campus of The University of Adelaide to produce the seed used for analysis.

Reciprocal F_1 grain, Sunco/Tasman, Tasman/Sunco, Gamut/Reeves and Reeves/Gamut, were produced by crossing 2 sets of parents. In each case the female was the first named cultivar in the cross. For analysis, three replicates of F_1 and parental grains from glasshouse-grown plants, each consisted of 5 mature grains, were dissected into embryo and de-embryonated portions (endosperm and seed coat) which were then compared with whole grains.

4.2.2 Apigenin di-C-glycoside analysis

Methods for extraction and quantification of ACGs were as reported by Asenstorfer et al. (2007) and Asenstorfer et al. (2006) with minor modifications, as explained in Chapter III, section 3.4.1.1.

4.2.3 Experimental design and data analysis

Three replicate extractions were conducted on each grain sample. Two peaks, previously identified as the isomers ACG1 and ACG2 (Asenstorfer et al. 2006), were quantified as rutin equivalents (Chapter 3 section 3.4.2.1). The data was corrected for losses during the SPE and drying steps based on the recovery of vanillin, the internal standard (Chapter 3 section 3.4.2.1).

The proportion of the 2 groups of Wessely-Moser regioisomers was expressed as a ratio, ACG1/ACG2. The amounts of ACG1, ACG2 and total ACG were expressed both

as concentration, μg/g, and as μg/grain. Concentration (μg/g) because it is relevant to the end-use of the grain and μg/grain because it was anticipated that it would be less affected by environmental factors. Environmental factors influence final grain weight and can confound genetic effects for grain constituents that are synthesized early in grain development.

The variation was analysed by one way ANOVA using GENSTAT version 14 (Chapter 3.4.3.2) with the variety and/or species included as treatment factors. Where significant differences were detected, Tukey multiple comparisons at 95% confidence interval was used to identify the different means.

For the field trial samples, the extractions were also conducted in 3 replicates. Coefficient of correlation, probability associated with the Student‟s t-test, and ANOVA were performed in GENSTAT software. The variety and location of the experiment were included as treatment factors. The variance of each treatment factor and their interactions were used in detecting genotype x environment (GxE) interaction. On the second set of analysis of GxE interaction, the 2008 data of grain ACG trait analyses was included as the third environment. The ANOVA of an unbalanced design was performed using GENSTAT.

4.3 Results and discussion

4.3.1 Apigenin di-C-diglycoside analysis

Extracts of grain of bread wheat (*T. aestivum*) cultivars and accessions of related species all contained the 2 major HPLC peaks representing the ACG diasterioisomers, referred to as ACG1 and ACG2, previously identified by Asenstorfer et al. (2006)

(Supplementary material 4.2). The regioisomers of ACG1 and ACG2 were not resolved by the HPLC system used in this investigation. A prior study (Asenstorfer et al. 2006) concluded on the basis of NMR data that ACG1 and ACG2 consisted of racemic mixtures of Wessely-Moser regioisomers. ACG1 had a retention time of 12.6 minutes and was previously shown to consist of a mixture of apigenin-6*C*-arabinoside-8*C*-glucoside (isoschaftoside) and apigenin-6C-glucoside-8*C*-arabinoside (schaftoside). The second peak with retention time 13.3 minutes, ACG2, was a mixture of apigenin-6*C*-arabinoside-8*C*-galactoside and apigenin-6*C*-galactoside-8*C*-arabinoside (Asenstorfer et al. 2006). No other peaks observed had absorption spectra consistent with apigenin di-*C*-glycosides. The ester bond in the ferulic acid and sinapic acid esters of ACG reported by (Asenstorfer et al. 2006) is broken during extraction with hydroxylamine, liberating the free phenolic acids and ACG. The ACG portion of the esters was therefore measured together with the free form.

4.3.2 Apigenin di-C-glycoside concentration and content per grain

4.3.2.1 ACG concentration

The frequency distribution for total ACG concentration in the 70 *Triticum aestivum* L. cultivars grown at Turretfield in 2005 approximated to a normal distribution with a range 43 - 166 μg (rutin equivalents)/g and a mean of 80 μg/g (Figure 4.1A). The concentrations of ACG1 and ACG2 in *T. aestivum* L. varied between 11 μg/g and 70 μg/g and between 29 μg/g and 105 μg/g respectively (Table 4.1). The ACG1, ACG2, and total ACG contents were consistent with data previously reported by (Asenstorfer et al. 2006).

The data for *T. aestivum* L. cultivars was subsequently compared with representatives of wild and cultivated species related to bread wheat. Whilst the former were grown in the field and the related species in a glasshouse, a small number of bread wheat cultivars that were grown in both environments were shown to have similar ACG content (supplemental material 4.3). As a consequence the comparison of the two sets of data would appear to be useful in assessing the relative ACG phenotypes of bread wheat and related species. Although only a limited number of accessions of each lower ploidy species were examined, the results suggest that the concentrations of ACG in *T. urartu* (AA), *T. monococcum* (A^mA^m), and *Ae. tauschii* (DD) were significantly lower ($p<0.05$) than *T. aestivum* (AABBDD), whereas *Ae. speltoides* (SS), *T. dicoccum* (AABB), *T. polonicum* (AABB), *T. turgidum* (AABB), *T.diccoides* (AABB), *T. carthlicum (*AABB*)*, and *T. durum* (AABB) were within the range observed for *T. aestivum* L. (Table 1). The low concentrations of ACG and in particular the glucose-containing isomers, ACG1, in grains of *T. urartu* and *T. monococcum* would suggest that the A genome would make only a minor contribution to ACG1 concentration in the higher ploidy species. There could be only 1 key enzyme in A genome that is limiting its contribution to ACG1 compared to B and D genomes. The other enzyme in the B and D genomes could be very active and have a significant contribution in the presence of both of these "very active enzyme" and the "limiting key enzyme" homeoforms in B and D genomes. As a consequence it seems likely that the ACG1 in the tetraploid and hexaploid species is contributed primarily by the B genome of *Ae. speltoides* and the D genome of *Ae. tauschii*. The full data set is available as Supplementary data 1.

Triticum spelta, another hexaploid wheat species, had the highest concentration ACG1, ACG2 and their total. There has been two tetraploid species proposed as candidates for

the progenitor of its A and B genomes, cultivated species *Triticum diccoccum* and wild species *Triticum diccoides*. The wild species has higher ACG concentration than the cultivated species. The D genome contributor of *T. spelta*, *Ae. tauschii*, has low ACG concentration. In comparison with the *Triticum diccocum*/*Aegilops tauschii* AUS22445 lines, which content and concentrations of ACGs were between the two parents, *T. spelta* has higher concentration of ACG1, ACG2 and their total.

4.3.2.2 ACG content per grain

There were significant differences ($p<0.05$) in 100 grain weight between and within species (Table 4.2). The total ACG concentration was weakly but significantly correlated with the 100 grain weight ($r = 0.121$, $p<0.05$). By comparison, there was a stronger and highly significant correlation between ACG content per grain and 100 grain weight ($r = 0.513$, $p<0.05$). The diploid ancestral species have significantly ($p<0.05$) lower 100 grain weight and ACG content per grain compared with the tetraploids and hexaploids.

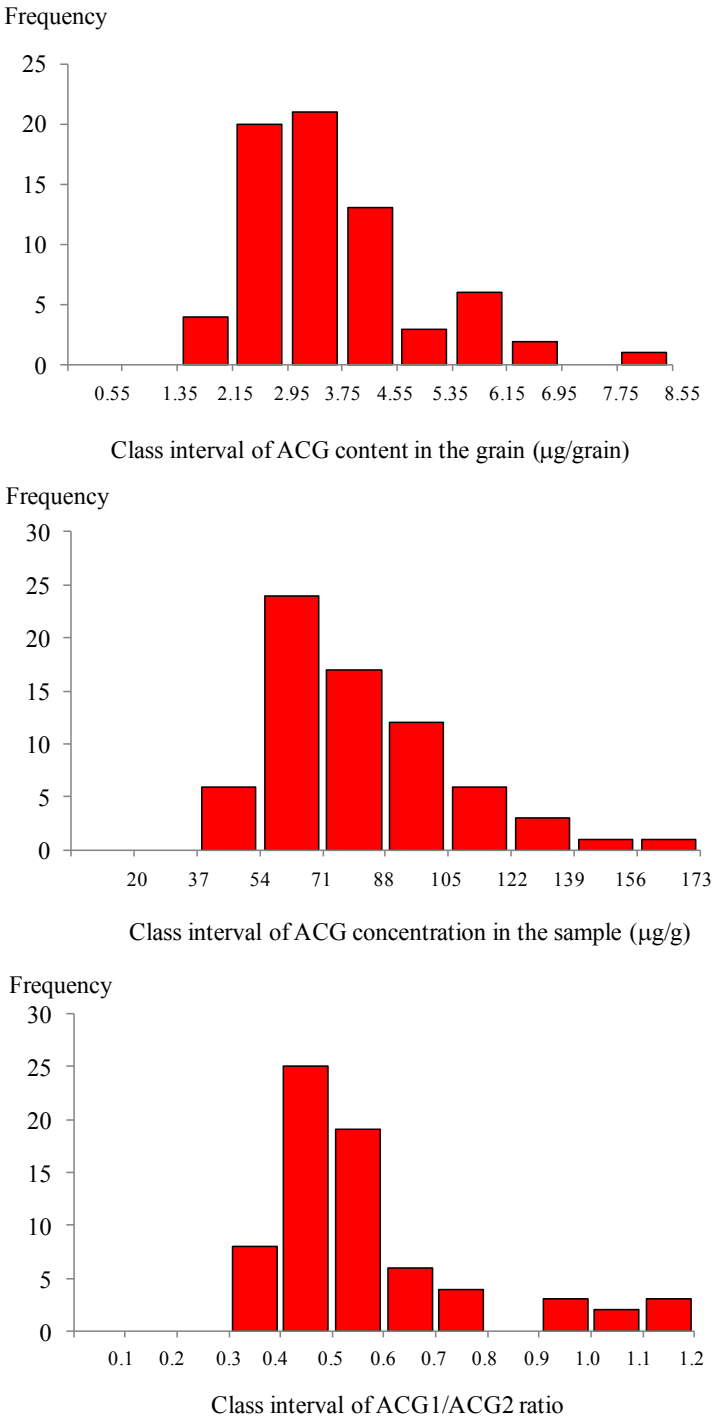

Figure 4.1. Frequency distribution for total apigenin di-*C*-glycoside (ACG) content and composition in grain of 70 bread wheat, *T. aestivum* L, cultivars grown at Turretfield, South Australia in 2005: A. Total ACG content in grain (µg/grain), B. Total ACG concentration (µg/g), and C. Ratio ACG1/ACG2.

Table 4.1 Range and means (± SE) for apigenin di-*C*-glycoside (ACG) content in grain of diploid, tetraploid, and hexaploid wheat species. The concentration and content are presented as the concentration (µg/g) and content in the grain (µg/grain) of each type of ACG (ACG1 and ACG2) and total ACG. The ACG1, ACG2, and total ACG data for all lines are available as supplementary material 1.

Species	Number of lines		Concentration per gram whole meal (µg/g sample)			Content per grain (µg/grain)		
			ACG1	ACG2	Total	ACG1	ACG2	Total
Triticum monococcum (A^mA^m)	n = 5	Max	4.2	22.4	26.5	0.1	0.6	0.6
		Min	1.2	12.3	15.8	0.0	0.3	0.3
		Means	2.5 ± 0.6	18.5 ± 1.9	21.0 ± 2.0	0.05 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
Triticum urartu (AA)	n = 5	Max	6.1	43.9	50.0	0.1	0.9	1.0
		Min	1.9	15.3	17.2	0.0	0.3	0.4
		Means	4.5 ± 0.7	33.9 ± 4.9	38.4 ± 5.6	0.1 ± 0.0	0.7 ± 0.1	0.8 ± 0.1
Aegilops speltoides (SS)	n = 5	Max	34.8	60.4	95.2	0.2	0.4	0.7
		Min	14.5	28.2	42.7	0.1	0.1	0.2
		Means	24.1 ± 3.7	42.5 ± 6.1	67.0 ± 9.5	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
Aegilops tauschii (DD)	n = 5	Max	17.9	22.0	38.7	0.3	0.3	0.6
		Min	9.2	11.1	20.4	0.1	0.1	0.2
		Means	13.4 ± 1.6	17.2 ± 2.0	30.6 ± 3.5	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
Triticum persicum (AABB)	n = 1		33.1 ± 1.9	72.2 ± 3.8	105.3 ± 5.7	1.1 ± 0.1	2.4 ± 0.1	3.5 ± 0.2
Triticum dicoccoides (AABB)	n = 1		31.0 ± 0.6	44.3 ± 0.8	75.3 ± 1.4	1.5 ± 0.0	2.2 ± 0.0	3.7 ± 0.1
Triticum dicoccum (AABB)	n = 3	Max	30.3	81.5	108.5	1.3	3.9	5.3
		Min	25.8	59.8	85.7	1.0	2.3	3.3
		Means	27.7 ± 1.3	73.1 ± 6.7	100.8 ± 7.6	1.2 ± 0.1	3.1 ± 0.5	4.3 ± 0.6
Triticum durum (AABB)	n = 5	Max	33.5	65.0	98.4	1.5	3.4	4.5
		Min	17.5	33.8	53.9	1.1	2.1	3.4
		Means	25.9 ± 3.0	51.7 ± 5.2	77.6 ± 7.4	1.4 ± 0.1	2.7 ± 0.2	4.1 ± 0.2
Triticum turgidum (AABB)	n = 1		26.7 ± 0.8	100.1 ± 3.4	126.8 ± 4.2	1.3 ± 0.0	5.0 ± 0.2	6.3 ± 0.2
Triticum polonicum (AABB)	n = 2	Max	44.6	67.0	111.5	2.4	3.6	6.0
		Min	24.1	38.6	62.7	1.2	1.9	3.0
		Means	34.3 ± 10.2	52.8 ± 14.3	87.1 ± 24.5	1.8 ± 0.6	2.7 ± 0.9	4.5 ± 1.5
Triticum spelta (AABBDD)	n = 1	Means	60.6 ± 2.6	118.8 ± 6.2	179.4 ± 8.7	2.6 ± 0.0	5.0 ± 0.1	7.6 ± 0.1
Triticum aestivum (AABBDD)	n = 70	Max	69.8	105.0	166.6	3.0	4.9	7.8
		Min	10.8	28.6	42.8	0.4	1.1	1.7
		Means	28.7 ± 1.4	51.7 ± 1.9	80.4 ± 3.0	1.3 ± 0.1	2.3 ± 0.1	3.6 ± 0.1

The frequency distribution for total ACG content per grain in the 70 field grown *T. aestivum* L. cultivars was bimodal with 83% less than 4.55 μg/grain and 17% greater than 4.55 μg/grain (Figure 4.1B). By comparison, the diploid *Aegilops* species would form a third group with ACG content less than 1.35 μg/grain while the tetraploid *Triticum* species would appear to be distributed within the range occupied by the bread wheats (data for each genotype in Supplementary 4.1). Polyploidisation and/or subsequent domestication and selection that have resulted in increased grain size (Table 4.1) appears to have been accompanied by increases in ACG concentration and in particular, ACG content per grain, the latter trait being up to 10 fold greater than the diploids.

Table 4.2 Means (+ SE) for 100 grain weight of the diploid, tetraploid, and hexaploid wheat. a-i: indicate significant differences ($p<0.01$) in 100 grain weight based on Duncan test of multiple comparison at 5% level.

Species	100 grain weight (g)
T. monococcum	2.7 ± 0.2 d
T. urartu	2.3 ± 0.1 e
Ae. speltoides	0.6 ± 0.1 g
Ae. tauschii	1.4 ± 0.1 f
T. dicoccum	4.8 ± 0.2 c
T. durum	6.3 ± 0.4 a
T. polonicum	5.3 ± 0.3 b
T. aestivum	6.1 ± 0.1 a

4.3.2.3 ACG content of reciprocal F_1 and parental grain

ACG1, ACG2 and total ACG in the de-embryonated fractions of grains (endosperm and seed coat) of parental genotypes, Sunco, Tasman, Reeves and Gamut, represented between 0 and 5.7 % of total grain ACG (Table 4.3). (Asenstorfer et al. 2006) reported that endosperm tissue did not contain any apigenin di-*C*-glycosides so presumably the ACG in the de-embryonated grains originated from the tissues of the grain coat (pericarp + testa). Ingram (2006) and Rathjen et al. (2008) reported low levels of ACG in the grain coat of developing wheat grains. By contrast with the parental genotypes, the amounts in the de-embryonated fractions of F_1 hybrid grains was significantly higher and represented between 29.8-53.9 % of the total in the grain (Table 4.3). These observations are difficult to explain. It was anticipated that the ACG phenotype would be similar to the female parent in each case given that the grain coat represents maternal tissue from the female parent used in the cross, besides the F_1 hybrids were grown together with their parents and their ACG analysis were performed at the same time. These results were subsequently confirmed in a second series of crosses, Reeves/Gamut and Gamut/Reeves, where the ACG content of the de-embryonated fraction of F_1 grains represented 41.8 and 48.2 % respectively of total grain ACG compared with only 0-5.7 % in the parents.

In the embryo fraction of F_1 grains, the amount of ACGs tended to be similar to the low ACG parent, except for ACG1 content of Tasman/Sunco. The similarity is consistent with the presence of different alleles, with the lower activity allele being dominant. This hypothesis would need to be confirmed by analysis of a segregating populations derived from one or more of these F_1s.

Table 4.3 Concentration, proportion and composition of the Apigenin di-*C*-glycosides (ACGs) in de-embryonated grain and embryo of F_1 reciprocal cross of Sunco-Tasman and Gamut-Reeves parent pairs.

Lines	ACG content (μg/grain)			% of total ACG in the grain	ACG composition (in ratio of ACG1/ACG2)
	ACG1	ACG2	Total		
De-embryonated grain					
Sunco	0.12 ± 0.02	0.05 ± 0.00	0.16 ± 0.02	4.75 ± 0.34	2.42 ± 0.30
Tasman	0.05 ± 0.00	0.05 ± 0.00	0.10 ± 0.00	2.14 ± 0.30	0.92 ± 0.09
Sunco/Tasman	2.53 ± 0.08	0.68 ± 0.08	3.21 ± 0.12	53.88 ± 3.12	3.84 ± 0.41
Tasman/Sunco	0.47 ± 0.07	0.55 ± 0.04	1.01 ± 0.10	29.84 ± 3.88	0.85 ± 0.08
Reeves	~	~	~	0.00 0.00	~
Gamut	0.19 ± 0.03	0.05 ± 0.01	0.24 ± 0.03	5.67 ± 1.91	3.54 ± 0.36
Reeves/Gamut	0.41 ± 0.00	0.90 ± 0.11	1.32 ± 0.11	41.82 ± 3.89	0.47 ± 0.06
Gamut/Reeves	1.41 ± 0.20	0.53 ± 0.03	1.93 ± 0.21	48.24 ± 2.79	2.67 ± 0.36
Embryo					
Sunco	1.85 ± 0.18	1.42 ± 0.16	3.27 ± 0.34	95.25 ± 0.34	1.3 ± 0.03
Tasman	1.71 ± 0.15	2.86 ± 0.29	4.57 ± 0.44	97.86 ± 0.30	0.6 ± 0.01
Sunco/Tasman	1.15 ± 0.17	1.66 ± 0.26	2.81 ± 0.43	46.12 ± 3.12	0.7 ± 0.01
Tasman/Sunco	1.04 ± 0.10	1.37 ± 0.10	2.41 ± 0.20	70.16 ± 3.88	0.8 ± 0.02
Reeves	0.67 ± 0.06	1.26 ± 0.13	1.92 ± 0.19	100.00 ± 0.00	0.5 ± 0.00
Gamut	2.34 ± 0.47	2.30 ± 0.49	4.63 ± 0.96	94.33 ± 1.91	1.0 ± 0.02
Reeves/Gamut	0.71 ± 0.07	1.14 ± 0.10	1.84 ± 0.17	58.18 ± 3.89	0.6 ± 0.01
Gamut/Reeves	0.79 ± 0.10	1.30 ± 0.22	2.10 ± 0.31	51.76 ± 2.79	0.6 ± 0.03
Whole grain					
Sunco	1.97 ± 0.20	1.46 ± 0.17	3.43 ± 0.36		1.35 ± 0.02
Tasman	1.75 ± 0.15	2.91 ± 0.29	4.66 ± 0.44		0.60 ± 0.01
Sunco/Tasman	3.69 ± 0.24	2.33 ± 0.32	6.02 ± 0.55		1.61 ± 0.12
Tasman/Sunco	1.50 ± 0.04	1.92 ± 0.07	3.43 ± 0.10	100.00	0.78 ± 0.02
Reeves	0.67 ± 0.06	1.26 ± 0.13	1.92 ± 0.19		0.53 ± 0.00
Gamut	2.53 ± 0.45	2.35 ± 0.48	4.88 ± 0.93		1.09 ± 0.03
Reeves/Gamut	1.12 ± 0.07	2.04 ± 0.08	3.16 ± 0.13		0.55 ± 0.03
Gamut/Reeves	2.20 ± 0.26	1.83 ± 0.24	4.03 ± 0.47		1.21 ± 0.09

4.3.2.4 ACG content of nullisomic-tetrasomic lines of Chinese Spring and Chinese Spring/*Triticum spelta* chromosome substitution lines

Results for total ACG (μg/grain) in nullisomic-tetrasomic lines of Chinese Spring were either generally inconsistent or not significantly different from Chinese Spring. Only in 5 instances were both individuals of a nullisomic pair similar to one another and significantly different from Chinese Spring. ACG content/grain in lines nullisomic for chromosomes 1B, 2B, 5A, and 5D (means ACG of 3.5, 2.8, 3.4, and 2.9 μg/grain respectively) were lower than Chinese Spring (mean ACG of 5.8 μg/grain) (supplemental material 4.4). None of the Chinese Spring nullisomic-tetrasomic lines contained zero ACG suggesting that variation in ACG content is associated with more than one gene. By comparison, in the Chinese Spring/*T.spelta* chromosome substitution series, only the line containing *T.spelta* 7B, with mean 9.1 μg/grain, had total ACG content per grain significantly greater ($p<0.05$) than Chinese Spring control (6.7 μg/grain), but similar to *T. spelta* (7.6 μg/grain) (supplemental material 4.4). The remaining Chinese Spring/*T.spelta* chromosome substitution lines were similar to Chinese Spring. Further investigation using populations derived from parents with contrasting total ACG phenotypes will be required to resolve the issue of number and location of genes involved in determining variation in total ACG.

4.3.2.5 Relative effects of genotype and environment on ACG content

A significant correlation was obtained for total ACG concentration (μg/g) of the 29 *T. aestivum* lines harvested from Urrbrae and Turretfield field trials in 2009 ($r = 0.544$, $p<0.05$). Similar data were analysed to estimate the proportion of observed variation explained by genotype (G), environment (E) and GxE. The genotype x environment interaction (GxE) was not significant while 34.7 % of the variation was due to

environment and 52.2 % was due to genotype. Significant correlations ($p<0.05$) were also obtained when comparing total ACG content per grain (μg/grain). For this trait, genotype x environment accounted for 13.2 % of the variation, while 5.0 % variation was due to environment and 74.2 % was due to genotype. The lower contribution from genotype when data was expressed as a concentration (μg/g) rather than per grain is possibly a reflection of the effects of environment during the later stages of grain filling impacting on final grain weight and greater or less dilution with starch. Comparison of genotypes grown in a broader range of environments and years would be required to define the relative contributions of genotype and environment to observe variation in ACG phenotype more accurately.

Significant correlations were also obtained for concentration and content of ACG1 and ACG2 (μg/g and μg/grain) of *T. aestivum* L. lines for grain harvested from Urrbrae and Turretfield. Again, genotype was the major source of observed variation. Similar results were also obtained when the ACG trait data from 2008 was included as one of the environmental condition. There was no significant GxE for any of the ACG traits, and genotype was the major source of variation.

4.3.3 Apigenin di-C-glycoside composition, ACG1/ACG2

The ratio of ACGs, ACG1/ACG2, indicates the relative proportions of *C*-glucosylation and *C*-galactosylation, specifically, a low ratio indicates a higher proportion of galactose containing ACGs. The ratio of ACG1/ACG2 in the grain of the *T. aestivum* L. genotypes sampled from Turretfield in 2005 varied between 0.3 and 1.2 (Figure 4.1C). The frequency distribution contained 2 distinct groups, a large group of cultivars (88.6 %) with low ACG1/ACG2 ratio (between 0.3-0.8) and a smaller group (11.4 %) with

high ACG1/ACG2 ratio (above 0.9). No cultivars were observed with ACG1/ACG2 ratio between 0.8-0.9. These results suggest the presence of different alleles at one locus associated with synthesis of ACG1 and ACG2. The mean ACG1/ACG2 ratios for Turretfield and Waite trials were strongly correlated (r = 0.83), indicative of a high heritability for this trait.

ACG1/ACG2 for *T. urartu* and *T. monococcum* ranged from 0.075 to 0.28, lower than for *T. aestivum* L. cultivars, whereas the ratio for *Ae. tauschii* was between 0.7 and 0.86. *Ae. speltoides* and the wild and cultivated tetraploid species were within the range for the main group of *T. aestivum* L. cultivars.

4.3.3.1 ACG composition and pedigree of *Triticum aestivum* L. cultivars

A high ACG1/ACG2 ratio for Sunco and Sunvale, backcross derivatives of the variety Cook, reported by Asenstorfer et al. (2006) was confirmed in this study (Supplemental material 4.1). However, other cultivars, Cunningham and Janz that also have Cook in their pedigree (Supplemental material 4.5) had a low ratio (0.5 and 0.7 respectively). The other parent of these low ratio cultivars can be traced back to Condor and 3-AG-3 (Martynov & Dobrotvorskaya 2006). Pedigree related cultivars with low ACG1/ACG2 ratio, Tasman, Suneca, Kennedy, and Hartog (Supplemental material 4.6) could be traced back to a low ratio cultivar, Ciano. The other parent of Tasman can also be traced back to Condor and 3-AG-3. There are 2 lines of Condor with different ratio phenotypes, 1.1 and 0.7 (supplemental material 4.1), while 3-AG-3 can be traced back to Chinese Spring (Sears, 1981 in Martynov & Dobrotvorskaya 2006), which is a low ratio variety (0.7, Supplemental material 4.1). These observations support the hypothesis that ACG1/ACG2 ratio is genetically controlled.

4.3.3.2 ACG1/ACG2 ratio in reciprocal F_1 grains and their parents

Analysis of whole grain, embryo and de-embryonated grain of parental lines and F_1 (Table 4.3) suggested that regulation of the ratio, ACG1/ACG2, may be different in embryo and non-embryo tissues of the grain. ACG1/ACG2 ratio for de-embryonated grain of the parental genotypes was greater than for embryos except for Reeves, where no ACGs were detected in the non embryo portion (Table 4.3). Embryos from the F_1 grains of Sunco/Tasman, Tasman/Sunco, Gamut/Reeves, and Reeves/Gamut have a low ACG1/ACG2 ratio similar ($p<0.01$) to the low ratio parents Tasman or Reeves (Table 4.3). This suggested that the ratio was genetically inherited, with the low ratio phenotype, associated with a higher proportion of *C*-galactosylation, being dominant. In the de-embryonated grain fractions of each reciprocal cross, the phenotype was more similar to that of the female parent used in the cross. This is consistent with the ACGs being located in the maternal grain coat tissue of the de-embryonated grain portion of F_1 grains. The ACG1/ACG2 ratios of the F_1 grain (whole grain, non-de-embryonated) were similar to the female parents.

4.3.3.3 ACG composition of nullisomic-tetrasomic lines of Chinese Spring

Analysis of the 42 nullisomic-tetrasomic lines of Chinese spring indicated that the pairs of stocks nullisomic for 1B, 7B, and 7D had ACG1/ACG2 ratios significantly different from the parent Chinese Spring. Lines lacking chromosome 1B or 7D had very low ACG1/ACG2 ratios similar to cultivars Ciano and Tasman, whereas for lines lacking chromosome 7B the ratio was higher than Chinese Spring and similar to cultivars Sunco and Cook (Figure 4.3). These results suggest that chromosomes 1B, 7B and 7D contain QTL associated with variation in synthesis of ACG1 or ACG2. Despite the substantial differences in ratio, the total amount of ACG per grain in lines lacking 7B or 7D were

not significantly different to the Chinese Spring parent except for the Nulli 7D-tetra 7B line (supplemental material 4.4). The similarity in phenotypes between the lines nullisomic for chromosome 7B and the high ratio wheat cultivars such as Sunco further suggests that these cultivars may have a null, non-functional or low activity allele at a QTL on chromosome 7B.

Elimination of chromosome 7B in Chinese Spring 7B nullisomic lines resulted in an increase in ACG1/ACG2, i.e. a relative increase in the glucose-containing isomer, possibly indicating the presence of a *C*-glycosyltransferase on 7B with specificity for UDP-galactose. A similar phenotype in the pedigree-related cultivars Cook, Sunco, and other high ratio cultivars could be explained by a deletion or mutation of a gene controlling this enzyme. The observation that high ACG1/ACG2 ratio was inherited as a recessive trait is consistent with this proposal. A doubled haploid population derived from a cross between Sunco and Tasman is examined in Chapter 6 to resolve this question.

The tetrasomic 7B lines have a lower ACG concentration than Chinese Spring, while the N7DT7A line has a similar ACG concentration. All other chromosome 7 nullisomic-tetrasomic lines have a higher concentration than Chinese Spring. This could indicate that there is a represser of ACG synthesis on chromosome 7B, with the tetrasomic lines of 7B having 2 copies, resulting in greater repression of ACG content.

Significant differences in ACG1/ACG2 ratio were also observed with lines lacking 4A and 5B chromosomes, however, the differences between these two lines and Chinese

Spring lines were very small. Nevertheless, this may indicate the presence of minor QTL for ratio on these chromosomes.

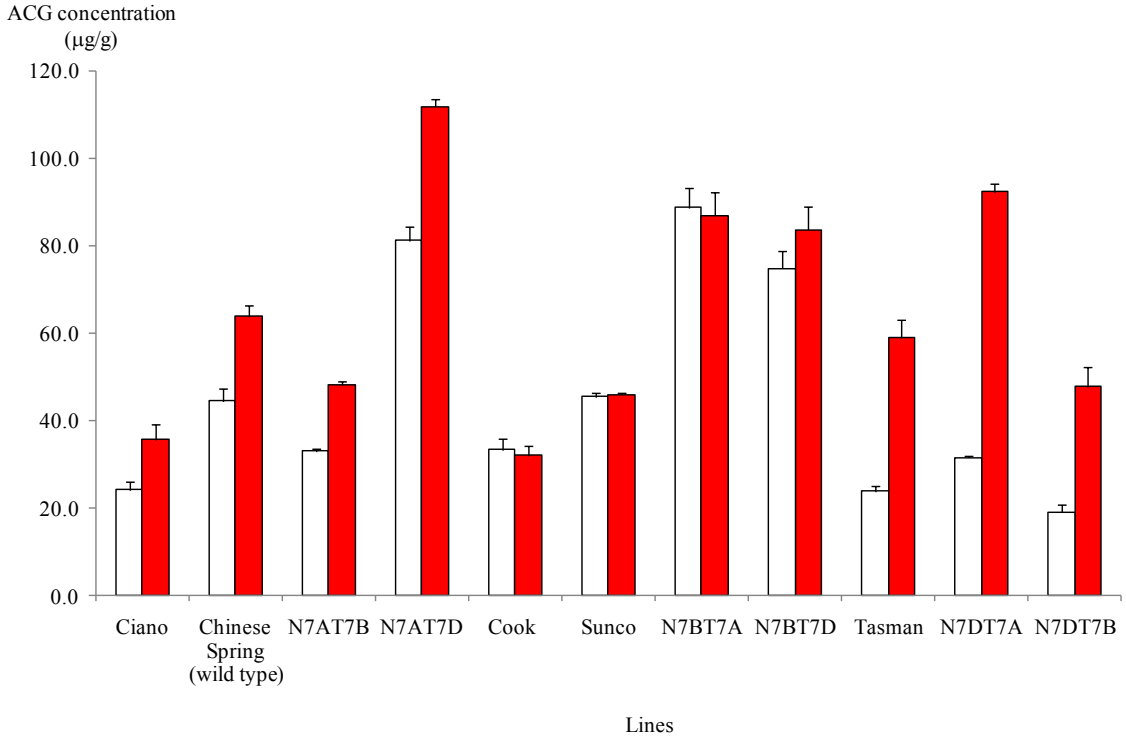

Figure 4.3 Content and composition of the 2 types of apigenin di-*C*-glycosides (ACG1 and ACG2) in the grain of the group 7 chromosome nullisomic-tetrasomic lines of Chinese Spring compared with Cook, Ciano, Tasman, and Sunco. The comparisons of the ACG1 and ACG2 content describe the differences in ACG1 to ACG2 composition in the grain. White bars represent ACG1, red bars represent ACG2. Bars represent SE of means.

4.3.3.4 Correlation among ACG components

The amounts of ACG1 and ACG2, showed a highly significant positive relationship ($r = 0.886$ and 0.952, $p<0.05$) with total ACG content. There was also a significant positive correlation between amounts of ACG1 and ACG2 although this correlation was

possibly confounded by the large proportion of genotypes with low ACG1/ACG2 ratio. ACG1 but not ACG2 or total ACG was significantly correlated with ACG1/ACG2 ratio. Control of the ratio therefore appeared to be independent of control of total ACG but depended on ACG1 concentration.

4.4 Conclusions

Significant genetic variation was identified for both total ACG content and ACG1/ACG2 ratio. Consequently, it should be possible to select wheat varieties with higher ACG content. Several observations indicate that genetic mechanisms that control total ACG content act prior to the glycosylation step in biosynthesis; namely the strong correlation between amounts of ACG1, ACG2 and total ACG, and the lack of significant correlation between ACG content and ACG1/ACG2. In addition, preliminary investigations of genotypes differing in total ACG content suggested that a lower content may be partly explained by a lower activity of enzymes such as chalcone isomerase, an enzyme catalyzing an early step in flavonoid biosynthesis (Kashem & Mares 2002). Variation in ACG content could reflect the relative activity of the enzymes located early in the flavonoid pathway, competition with other branches of the pathway for flavone precursors and/or UDP sugars, or possibly regulation of flavonol-2-hydroxylase, the first committed step in the proposed branch in the flavonoid biosynthesis pathway leading to ACG.

The ACG ratio, whilst possibly not of interest commercially, provides an avenue towards understanding the mechanisms and regulation of *C*-glycosylation. No ACGs containing the same sugar residue at both C6 and C8 were identified and all ACG isomers contained arabinose attached to either C6 or C8. Since changing the ratio did

not affect total ACG content, it seems possible that the proportions of ACG1 and ACG2 depended on either the relative sizes of the available pools of UDP-glucose and UDP-galactose, the activity of the epimerase that catalyses their interconversion or variation in the activity and specificity of the *C*-glycosyltransferases. Different alleles of genes coding for *C*-hexosyltransferase may result in differences in UDP-sugar recognition. Elimination of chromosome 7B in Chinese Spring 7B nullisomic lines resulted in an increase in ACG1/ACG2, i.e. a relative increase in the glucose-containing isomer, possibly indicating the presence of a *C*-glycosyltransferase on 7B with specificity for UDP-galactose. A similar phenotype in the pedigree-related cultivars Cook, Sunco, and Sunvale and other high ratio cultivars could be explained by a deletion or mutation of a gene controlling this enzyme.

Title of Thesis: Genetic control of Apigenin di-*C*-glycosides biosynthesis in bread wheat (*Triticum aestivum* L.) grain and their potential as yellow pigment of Asian alkaline noodles

Student Name: Grace Yasmein Wijaya

Pg # PhD

Chapter V

Quantitative assessment of the contribution of Apigenin di-C-glycosides (ACG) in determining the colour of yellow alkaline noodles (YAN) in comparison to that of Lutein

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STATEMENT OF AUTHORSHIP

Title of Paper Quantitative assessment of the contribution of Apigenin di-C-glycosides (ACG) in determining the colour of yellow alkaline noodles (YAN) in comparison to that of Lutein

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Name of candidate Grace Yasmein Wijaya

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Designed and developed the detail experiment for data collection, performed the biochemistry analysis on all samples (excluded HPLC machine set-up), performed the Buhler and Quadrumate Junior milling, performed noodle and miiling data collection, analyzed and interpreted the data, planned the article, wrote manuscript and acted as corresponding author

Certification that the statement of contribution is accurate

Signed ....*Date*..... 19 March 2012

co-author name Dr. Robert A. Asenstorfer

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Co- supervisor of the project; developed provided ongoing input relating to the separation and quantitation of ACGs by HPLC, noodle making and data collection by reflectance spectrometer; set-up the HPLC machine prior the sample analysis, provided critical evaluation on the initial data analysis on the correlation of yellow colour of noodles and yellow pigment (ACG and lutein) content.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed ....*Date*..... 20.3.12

co-author name Assoc. Prof. Daryl Mares

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Principal supervisor of the project, developed the initial outline for the project, provided background IP for the project, all the germplasm and genetic stocks requested, provided critical evaluation, supervised development of work and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed ..... *Date* 20/3/2012.

Chapter V: Contribution of Apigenin di-*C*-glycoside (ACG) to the colour of yellow alkaline noodles (YAN) in comparison to that of Lutein

5.1 Introduction

Yellow alkaline noodles (YAN) are an important end-product for Australian wheat throughout South East Asia and Japan (QDPI 2009). The yellow colour is contributed by xanthophylls, primarily lutein and its fatty acid esters, apigenin di-*C*-glycosides (ACG1 and ACG2, Figure 2.2) and yellow colour additives used by the manufacturer (Asenstorfer & Mares 2006, Asenstorfer et al. 2006, Fortmann & Joyner 1978, Morris et al. 2000, Wang 2001, Ward et al. 1995). Xanthophylls are present at relatively low concentration in bread wheat and give colour ranging from white to pale yellow to noodle dough (Parker et al. 1998). By contrast, ACGs remain colourless in water or acid solution and give the yellow colour only after alkaline solution is added during the preparation of YAN (Asenstorfer et al. 2006). Raising the levels of the endogenous compounds that contribute to the yellow colour could reduce, and potentially eliminate the need for colour additives and add value to Australian wheat. In particular, raising the ACG content could improve the YAN colour derived from natural endogenous compounds without impacting on the colour of flour or a range of other wheat-based foods such as bread that are traditionally creamy to white in colour.

The contribution of xanthophylls to noodle colour is well established but there is only limited information for ACGs. Typically, for YAN prepared with alkaline salts but no colour additives, the increase in CIE (Commission Internationale l‟Eclairage) b*

compared with control white salted noodles (WSN) is around 6 units (Wijaya et al 2009). However, how much of the yellow colour developed in the presence of alkaline salts is due to ACGs has not been quantified. By comparison, the b* of commercial samples of YAN sourced from the supermarket can be as high as 45 – 55 (Wijaya et al. 2010) of which 20 – 30 b* units would appear to be due to the presence of additives such as tartrazine and sunset yellow. In this context it is not clear whether there is sufficient genetic variation for ACG content to firstly, allow breeding wheat varieties for better YAN colour and secondly, to estimate how much ACG would be required to significantly reduce or eliminate the need for colour additives.

Previous studies have relied on a spectrophotometric assay for ACG but these results have recently been shown to be confounded by interaction between the extractant and flour constituents and therefore not specific for ACGs (Asenstorfer & Mares 2006). In addition, the ACGs appear to be located in the embryo and seed coat (Fortmann & Joyner 1978, Ingram 2006, Miskelly 1996, Rathjen 2006), which would limit their recovery in flour during the milling process.

The aims of this study were to:

- compare the lutein and ACG content of grain and flour in a range of bread wheat genotypes
- determine the relationships between these traits and both total YAN yellow colour and the component of yellow colour developed specifically in the presence of alkaline salts
- estimate the impact of the available genetic variation for ACG content on improving the yellow colour of YAN, and

- compare the recoveries of ACG and lutein in flour.

To achieve these aims, the lutein and ACG content of grain and flour were compared, and their correlation with both the total yellowness of YAN and the yellow colour developed specifically in the presence of alkaline salts examined. In addition, the contribution of ACG to the yellow colour of YAN was predicted using a standard curve of YAN b* versus added rutin and compared the observed change in b* due to the presence of alkali. In turn, the potential increase in colour if all the grain ACG could be recovered in flour was calculated. The effects of milling were determined by comparing grain and flour lutein and ACG content and compared in relation to the grain tissue locations of these substances.

5.2 Materials and Methods

5.2.1 Plant materials

Grain of twenty eight Australian commercial bread wheat (*Triticum aestivum* L.) cultivars representing all the wheat production zones in Australia was produced in a field trial at Turretfield, South Australia in 2005 and stored at low humidity and room temperature in sealed containers. Cultivars and their region of origin are: Ventura, Sunvale, Sunco and Sunlin (Northern New South Wales); Diamondbird (Southern New South Wales), Cunningham, EGA Gregory, Hartog, Janz, EGA Hume, Kennedy, Lang, Batavia and Baxter (Queensland); Spear, Frame, Halberd, Krichauff and Kukri (South Australia); Chara, Meering, Silverstar and Annuello (Victoria); GBA Ruby, Cascades, Carnamah, Reeves, and Westonia (Western Australia). To provide sufficient sample size for Bühler Milling, nine of these cultivars from 2005 (Sunvale, Sunco, Hartog,

EGA Hume, Halberd, Krichauff, Silverstar, GBA Ruby and Reeves), were grown as larger plots in Turretfield, in 2009.

Grain of a large bulk of cultivar Suneca were used as standard throughout the flour milling experiments, while a bulk flour sample of cultivar Sunvale was used to quantify the effect of addition of different amounts of rutin on YAN colour.

5.2.2 Preparation of flour and noodles

5.2.2.1 Grain milling

Three experimental mills were used: Udy cyclone mill, Quadrumat Junior mill (Brabender, Germany), and Bühler mill (Bühler Ag, Switzerland) depending on the sample size and/or the need for milled flour. The Udy cyclone mill was used to prepare whole meal flour for analysis of total grain content of ACG or lutein. The Quadrumat Junior mill requires at least 50g of grain samples, and is suited to the rapid and simple preparation of flour substantially free from bran and pollard in a single step. The Bühler mill is a more sophisticated method than the Quadrumat mill, capable of milling up to 5 kg grain, and separating the bran and pollard fractions from the flour in a progressive particle size reduction and sieving process.

In this experiment, the Udy cyclone mill fitted with a 0.1 mm screen to control particle size was used to grind the grain samples to a fine whole meal (Asenstorfer et al. 2006). A Quadrumat Junior mill was used for small samples from the 2005 trial whereas the Bühler mill was used to prepare higher quality flour from the 2009 samples. The flour yields from the Quadrumat Junior and Bühler were in the range of 65%-72% and 60%-71% respectively. For the Quadrumat Junior, 150g was weighed and pre-conditioned

overnight to 15% moisture prior milling. Samples of Suneca were milled every 7 samples to monitor the performance of the mill. In the Bühler experiment, 5kg of grain was preconditioned to 15% overnight prior to milling and pooled break, pooled reduction, Bran and pollard fractions collected. Subsequently, the pollard fraction was re-sieved to remove residual flour and retain a sieved pollard fraction.

5.2.2.2 Preparation of noodle sheets

YAN noodle sheets were prepared as described by Asenstorfer et al. (2006). Ten grams of flour was mixed with 3.5 mL of sodium carbonate solution (2% w/v), in a small stainless steel bowl using a paddle attached to an electric drill press. The mixture was formed into a sheet by passing through a hand operated spaghetti machine several times.

To estimate the proportion of the yellow colour of YAN, which develops in the presence of the alkaline conditions, white salted noodles (WSN) were used as a control. WSN were prepared in the same way as YAN except that sodim carbonate was replaced with sodium chloride. The YAN and WSN sheets and noodles obtained are shown in Figure 5.1.

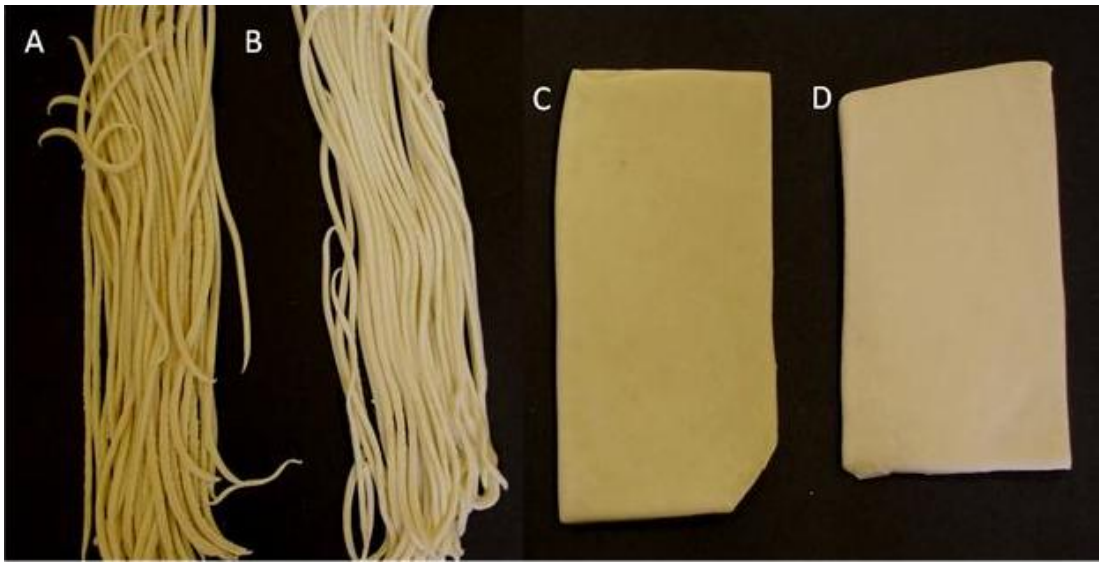

Figure 5.1 Noodles and noodle sheets for measurements of b* and Δb*. A. YAN B. WSN C. YAN sheet. B. WSN sheet.

5.2.3 Measurement of colour of YAN and WSN

The colour of YAN and WSN, was measured by CIE (Commission Internationale l"Eclairage) L*, a*, b* (Humphries et al. 2004, Oliver et al. 1992, Wyszecki & Stiles 1982) using a Dataflash 100 reflectance spectrophotometer (Datacolour International, USA) at 0, 2, 4, and 24 hours. Initially, this instrument was calibrated with a white tile and a black card.

L* measures changes in brightness on a 0 to 100 scale where 0 = black and 100 = white, whilst a* measures variation in red-green colour range. The values of a* are close to zero for wheat flour and therefore can effectively be ignored. The values of CIE b* measure the yellow colour, which range from -60 (pure blue) to +60 (pure yellow). The colour measurements for each noodle sheet were determined as the mean of duplicate

measurements. The amount of yellow colour developed specifically in response to the alkaline pH was estimated as Δb* (YAN b*- WSN b*).

5.2.4 Analyses of yellow pigment content in grain and flour

5.2.4.1 ACG analysis of grain and flour

Extraction and quantification of ACGs of grains were performed according to Asenstorfer et al. (2007) and Asenstorfer et al. (2006) with minor modifications described in Chapter III section 3.4.1.1. A larger sample size, 0.5g, was extracted with 5 mL of 0.1 M hydroxylamine (Sigma, St Louis, USA) pH 7.0, spiked with 15μL of vanillin standard (Sigma, St Louis, USA), mixed for 24 hours prior centrifugation at 2100g for 10 minutes, then half the supernatant loaded onto a reverse phase SPE column (Strata^{TM} -X33μm C18 polymorphic sorbent, Phenomenex, Torrance, CA, USA) that had been preconditioned with methanol and H_2O. The fraction containing ACGs eluted from the column was spiked with 50μL rutin, vacuum concentrated and the residue resuspended in 1mL methanol (50%) for HPLC analysis. The rest of the method was as described in section 3.4.1.1.

ACG content and composition traits were calculated as described in section Chapter 3, section 3.4.2.1. The recovery of ACG in Quadrumat Junior flour was calculated as a percentage of total grain ACG. For Bühler Mill, the proportion of ACG in the flour were calculated as the total ACG in reduction and break flour fractions as a percentage of the total ACG in all mill fractions, flour, bran and pollard. The ACG content in each fraction was calculated as the concentration of total ACG multiplied by the amount of the fraction recovered from milling.

5.2.4.2 Lutein analysis of grain and flour

5.2.4.2.1 Lutein extraction and analyses

Lutein was extracted using a sequential method (Soriano et al. 2007) that was optimized by Raoufi-Rad (2007). The whole meal samples (2 g) were extracted sequentially, firstly with 10mL of methanol/tetrahydrofuran (1:1 v/v) containing 0.1% butylated hydroxytoluene (BHT) and secondly with hexane, each for 2 hours, under nitrogen at 28^0C in a temperature regulated shaker. During the first extraction, β-apo-8‟-carotenal (50 μL) was added as internal standard. After each extraction, centrifugation was performed for 10 minutes at 3500 rpm following shaking, and the supernatant then was transferred to a glass tube. The supernatants were pooled and then vacuumed dried and purged with a stream of high purity nitrogen prior to storing for HPLC analysis. The dried residues obtained were reconstituted with 1 mL methanol/*tert*-butyl methyl ether (MTBE, 1:1 v/v), vortexed for 30 second. Quantification was performed by reverse-phase HPLC analysis using C30 reversed-phase column protected by a C18 guard column (Breithaupt et al. 2002). The mobile phase consisted of two solvents, A (81:15:4 methanol/MTBE/water) and B (90:10 MTBE/methanol). They were used at a flow rate of 1 mL/min with following protocol: 100% A for 10 minutes, 0-50% B for 40 minutes, 50-100% B for 10 minutes, 0-100% A for 5 minutes and an isocratic elution with 100% A for 5 minutes to condition the column (Breithaupt et al. 2002) modified according to Raoufi-Rad (2007). Absorbance at wavelengths 445 was used in detection and quantification of lutein. The lutein peak was detected at around 7 minutes, while peaks of the monoester and diester forms of lutein were detected at around 32-34 minutes and 44-46 minutes respectively, which were verified by their spectrums.

5.2.4.2.2 The use of internal and external standard solutions in lutein analyses

The use of β-apo-8'-carotenal as internal standard

The β-apo-8‟-carotenal was used as internal standard to enable correction of data for losses during the extraction. Initially, the standard β-apo-8‟-carotenal stock solution was made up from 5mg powder in 1 mL methanol/MTBE (1:1 v/v)). This solution was filtered by using filter paper (Whatman no 541, diameter 55mm). A serial dilution of this standard were mas and the absorbances of these dilutions were measured by using spectrofotometer (at 451nm wavelength). The dilution with 0.5 absorbance was used as internal standard. The concentration of this β-apo-8‟-carotenal standard was calculated by using Lamber-Beer Law:

$$A = [C] \times \varepsilon \times l$$

where:

A = absorbance (= 451nm).

ε = coefficient of extinction of β-apo-8‟-carotenal (1% g/mL = 139,500 at 451nm).

l = the path of the wavelength (= 1cm cuvet length).

Two replicates of controls contained blank samples (without whole meal or flour materials) were spiked with 50 µL of β-apo-8‟-carotenal standard stock solution. The analytical 450nm UV wavelength detected the peak of β-apo-8‟-carotenal standard at about 18 to 20 minutes, which was verified by its spectrum. The areas of the β-apo-8‟-carotenal peaks obtained from these controls and from the samples were then compared and calculated to get the β-apo-8‟-carotenal corrected area values as follows:

$$\frac{\text{area of } \beta\text{-apo-8‟-carotenal pure std}}{\text{area of } \beta\text{-apo-8‟-carotenal std in each samples}} \times \text{Area of lutein}$$

The use of xanthophylls to construct the standard curves

Th lutein standard curves were made by a serial dilutions of 1mg/mL xanthophylls in MTBE, from 1 to 100 times. The areas of these xanthophyll standard dilutions were then measured by HPLC at 445nm wavelength. The xanthophyll concentrations were plotted as y axis, while the areas as x axis in Microsoft Excell worksheet (version 2007). A trendline and the coefficient of this standard curve were regenerated to calculate the lutein content in the flour and whole meal samples.

The lutein content in the 2g samples with 1mL final dilution in MTBE (1:1 v/v) was calculated follows:

$$\frac{\beta\text{-apo-8‟-carotenal corrected area}}{\text{standard curves coefficient}} \times \frac{1000}{\text{sample weight (g)}}.$$

In the absence of authentic lutein ester standards, the amounts of mono- and di-esters were expressed as μg lutein equivalents. For the purposes of this investigation, total lutein (lutein + lutein mono- and di-esters) was calculated.

5.2.5 Estimation of ACG contribution to yellowness of YAN

Since pure apigenin di-*C*-glycosides were not available commercially, a standard curve was constructed using a closely related compound, rutin (an apigenin-*C*-glycoside).

Noodle sheets were prepared from Sunvale flour with the addition of rutin at 8.3, 16.6, 31.3, 62.5, 125, 250, 500, and 1000μg/g according to the method described in section 5.2.2.2. Control noodle sheets were made from the same flour without any rutin addition. The b* of the noodle sheets were measured at 0, 2, 4, and 24 hours as mentioned in the section 5.2.3. The Δb* was calculated as b* of the noodles with added rutin – b* of control noodles.

To estimate the ACG constribution to Δb*, standard curve was constructed by plotting the rutin concentration that added to the noodle sheets against Δb*. This was performed for each time measurements (0, 2, 4 and 24 hours) to follow the effect of each rutin addition into noodle dough to the change in the yellowness of the noodles.

5.2.6 Data analysis

The variation in the ACG and lutein content were analysed by one way ANOVA with GENSTAT 14 (Chapter 3 section 3.4.2.2) with the variety included as treatment factor. When significant differences were detected, LSD at p=0.05 was used to identify the different means.

The flour recovery from Quadrumat Junior milling was calculated as percentage of the weight of the starting material. Meanwhile, the flour recovered from Bühler milling was calculated as percentage of break flour and reduction flour per total grain weight. Correlations between ACG content or, (%) recovery in flour, and YAN (b*) or YAN Δb* were determined by simple regression analysis using GENSTAT software (version 14; VSN International, Hemel Hempstead, UK). The b* or Δb* was the response variate while the ACG content or (%) recovery in flour was the explanatory variate.

GraphPad Prism (version 5.04; GraphPad Software, Inc., CA, USA) was used to plot the curve relating change in YAN b* (Δb*) to amount of added rutin to wheat flour. Δb* data at 0, 2, 4, and 24 hours was plotted as non-linear regression curves. The one site-specific binding of Binding-Saturation equation was used to generate the relationship between rutin concentration and YAN Δb*:

$$Y = a*X/(b + X)$$

Y is the Δb* of YAN, X is the concentration of rutin (μg/g) while a and b are constants.

5.3 Results and Discussion

5.3.1 ACG and lutein content in wheat grain and flour

The mean ACG concentration in grain was 71.6μg/g (Table 5.1) while the concentration in flour was 19.3μg/g. By comparison, the mean lutein concentrations in wheat grain and flour were similar to each other, 1.086μg/g and 1.011μg/g respectively, and much lower than ACG concentrations.

The average recovery of lutein in Quadrumat Junior mill flour was 90% (Table 5.1) and there was a very strong correlation (r = 0.941) between the lutein concentrations in grain and flour. By contrast, only 30%, on average, of grain ACG was recovered and there was no correlation between grain and flour ACG concentration (r = -0.007). Thus, not only was the recovery of grain ACG in flour very low, but the amount recovered in flour was seemingly not determined by the grain ACG concentration but rather by the milling performance of the wheat.

5.3.1.1 The effect of milling on the proportion of ACG and lutein in relation to their tissue location in the grains

The composition of the flour recovered during milling reflects firstly, how cleanly the starchy endosperm is separated from the other tissues comprising the grain, and secondly, the tissue location of grain constituents. Earlier work reported by Asenstorfer & Mares (2006) indicated that ACGs were concentrated in the embryo or germ tissue with lower concentrations in the grain coat but seemingly none in the starchy endosperm. These observations were later confirmed by Ingram (2006). It was concluded therefore that the ACG content in flour was dependent on the level of contamination of flour by germ and/or bran. Flour ACG1 as a proportion of the total flour ACG was higher than in grain with means of 44.6% for flour and 32.4% respectively, while the proportion of ACG2 in the grain is higher than in the flour with means 67.6% and 55.4% respectively (Table 5.2 and supplemental materials 5.2 for the details). These differences were reflected in the ACG1/ACG2 ratios, which were higher in flour than in the grain, 0.6-1.3 in flour (mean = 0.8) and 0.3-1.0 (mean = 0.5) in the grain. The results indicate that more ACG2 than ACG1 is removed with the bran and pollard during milling and imply that the proportions of ACG1 and ACG2 in grain coat and embryo tissues are different. Furthermore, these observations could provide a clue to which grain tissue, embryo or grain coat, is the main source of the ACG in flour. Currently there is insufficient data available to allow resolution of this question.

The xanthophylls present in the grain were predominantly in the form of free lutein (26.4-40.6%, mean = 34) and its mono- (25.3-44%, mean = 34) and di-fatty acid esters (15.4-41.5%, mean =32) (supplemental material 5.3). By comparison, the proportions of

the different lutein species in Quadrumat Junior mill flour were: free lutein (17.9-32.2%, mean = 25), mono-esters (27.2 – 37.8, mean = 34) and di-esters (31.8 – 53.8, mean = 41). Thus the proportion of total lutein present as free lutein in the flour was significantly lower ($p<0.01$) than the proportion in grain and conversely the proportion present as di-esters was significantly ($p<0.001$) higher than grain. There appeared to be no significant difference ($p<0.05$) in monoesters.

Previous studies reported that there were little or no esters in grain at maturity but that esters form during grain storage depending on temperature and humidity (Kaneko et al. 1995, Kaneko & Oyanagi 1995). The high proportion of ester forms in the grain and flour in this study is presumably a reflection of the grains having been stored at low humidity since 2005 prior the experiment in 2009.

Lutein was distributed almost evenly in the grain tissues according to Hentschel et al. (2002), however, by contrast, Law (2005) reported higher concentrations of lutein in the embryo (up to 6 time the concentration in endosperm) than in other tissues. However, as the embryo only represents 3% of the grain weight and most is removed by milling, much of the lutein recovered in the flour originates from endosperm tissue (Soriano et al. 2007). Law (2005) further reported that esters were present primarily in the endosperm and seed coat whilst there appeared to be only very low amounts in the embryo. These observations might explain the differences in the proportions of free lutein and ester forms in flour compared with the grain. During milling it is anticipated that most of the embryo tissue with its higher free lutein concentration would be discarded in the germ fraction.

Table 5.1 Total ACG and lutein concentrations in grain and flour and the percentage recovery in Quadrumat Junior mill flour.

Cultivars	Total content in grain (μg/g)		Total content in flour (μg/g)		% Recovery	
	ACG	Lutein	ACG	Lutein	ACG	Lutein
Ventura	71 ± 2.5	0.86 ± 0.03	16 ± 0.1	0.79 ± 0.02	22 ± 0.7	92 ± 0.6
Sunvale	81 ± 0.2	0.76 ± 0.08	14 ± 1.0	0.66 ± 0.00	18 ± 1.2	89 ± 3.1
Sunco	95 ± 4.8	0.74 ± 0.02	16 ± 1.4	0.58 ± 0.03	17 ± 1.3	79 ± 1.6
Diamondbird	51 ± 2.8	0.95 ± 0.01	11 ± 1.6	0.80 ± 0.03	22 ± 2.4	85 ± 1.7
Sunlin	78 ± 6.6	0.99 ± 0.04	17 ± 0.6	0.95 ± 0.02	23 ± 1.2	96 ± 0.5
Cunningham	92 ± 3.2	1.00 ± 0.06	18 ± 0.8	0.87 ± 0.03	19 ± 0.2	87 ± 2.2
EGA Gregory	53 ± 3.4	1.02 ± 0.04	13 ± 0.6	0.93 ± 0.02	25 ± 2.3	91 ± 0.8
Hartog	92 ± 14.9	1.05 ± 0.05	17 ± 0.3	0.76 ± 0.01	20 ± 2.8	72 ± 1.4
Janz	72 ± 4.9	0.88 ± 0.02	20 ± 2.0	0.86 ± 0.03	28 ± 2.7	97 ± 1.9
EGA Hume	68 ± 1.6	0.83 ± 0.03	19 ± 1.2	0.75 ± 0.01	28 ± 1.4	91 ± 0.5
Kennedy	60 ± 7.8	0.82 ± 0.03	17 ± 0.8	0.71 ± 0.00	29 ± 4.6	86 ± 1.0
Lang	105 ± 9.6	0.72 ± 0.04	19 ± 1.4	0.70 ± 0.02	19 ± 3.2	98 ± 2.0
Batavia	55 ± 3.8	1.18 ± 0.03	15 ± 1.7	1.18 ± 0.00	28 ± 4.5	100 ± 0.8
Baxter	63 ± 2.8	0.76 ± 0.04	16 ± 0.9	0.66 ± 0.02	26 ± 1.5	88 ± 1.5
Spear	76 ± 4.2	1.05 ± 0.03	17 ± 1.2	1.03 ± 0.01	22 ± 2.4	98 ± 0.7
Frame	93 ± 8.6	1.01 ± 0.05	19 ± 0.7	1.16 ± 0.00	20 ± 1.3	90 ± 0.7
Halberd	109 ± 4.6	1.10 ± 0.05	25 ± 2.3	1.06 ± 0.05	22 ± 1.4	88 ± 0.9
Krichauff	65 ± 1.4	1.97 ± 0.03	21 ± 0.2	2.00 ± 0.03	32 ± 0.4	98 ± 0.4
Kukri	83 ± 5.1	1.16 ± 0.06	21 ± 1.1	1.06 ± 0.00	25 ± 0.7	92 ± 1.9
Chara	54 ± 3.5	1.01 ± 0.17	18 ± 0.6	1.21 ± 0.09	34 ± 2.8	96 ± 0.5
Meering	78 ± 2.5	1.04 ± 0.03	28 ± 0.6	1.23 ± 0.05	37 ± 1.7	99 ± 1.2
Silverstar	43 ± 1.8	0.95 ± 0.02	25 ± 1.1	0.82 ± 0.03	59 ± 2.7	86 ± 1.9
Annuello	56 ± 4.4	1.37 ± 0.05	21 ± 2.7	1.13 ± 0.06	39 ± 5.8	84 ± 3.6
GBA Ruby	81 ± 6.9	0.95 ± 0.05	22 ± 1.2	0.57 ± 0.03	28 ± 2.4	61 ± 2.6
Cascades	62 ± 6.4	1.95 ± 0.04	21 ± 1.1	1.84 ± 0.01	35 ± 5.3	95 ± 0.5
Carnamah	63 ± 8.1	1.43 ± 0.07	27 ± 1.2	1.26 ± 0.01	45 ± 7.0	89 ± 0.5
Reeves	43 ± 0.9	1.03 ± 0.09	27 ± 2.1	0.94 ± 0.05	61 ± 6.2	92 ± 1.1
Westonia	62 ± 3.4	1.86 ± 0.02	20 ± 1.4	1.80 ± 0.00	32 ± 3.3	97 ± 2.1
Max	109	1.97	28	2.00	61	100.0
Min	43	0.72	11	0.57	17	61.0
Means	72 ± 3.3	1.09 ± 0.10	19 ± 0.8	1.01 ± 0.10	29 ± 2.0	89.8 ± 1.6

Table 5.2 Proportion of total yellow pigments in flour compared with grain; range and means for 28 bread wheat cultivars. The ACGs are presented as ACG1 and ACG2 while the luteins are presented as lutein, lutein-monoester, lutein-diester and total lutein-ester.

Pigment	Proportion in grain (%)			Proportion in flour (%)		
	Max	Min	Means	Max	Min	Means
ACG1	49.7	24.8	32.4 ± 3.4	57.0	36.2	44.6 ± 3.8
ACG2	75.2	50.3	67.6 ± 3.4	63.8	43.0	55.4 ± 3.8
ACG1/ACG2 Ratio	1.0	0.3	0.5 ± 0.1	1.3	0.6	0.8 ± 0.1
Lutein	40.6	26.4	34.1 ± 0.6	32.2	17.9	25.2 ± 0.7
Monoester Lutein	44.0	25.3	34.0 ± 0.8	37.8	27.2	33.8 ± 0.5
Diester Lutein	41.5	15.4	31.9 ± 1.2	53.8	31.8	41.0 ± 1.0
Total Lutein-ester	73.6	59.4	65.9 ± 0.6	82.1	67.8	74.8 ± 0.7

5.3.1.2 Comparison of Bühler and Quadrumat Junior Mills

As mentioned in the Materials and Methods section, the Bühler mill is a more sophisticated experimental flour mill than the Quadrumat Junior mill and therefore likely to provide a better indication of the way a wheat variety will perform in a commercial flour mill. It was therefore of interest to determine whether the Bühler gave a greater recovery of ACG and whether there was a stronger relationship between grain and flour ACG concentration.

Total ACG concentration in Bühler flour ranged from 6.9-33.5μg/g, with a mean of 18.3 μg/g (Figure 5.3 and supplementary material 5). This was not significantly ($p<0.001$) different from that obtained with Quadrumat Junior Mill and perhaps more significantly, one mill was not consistently better or worse than the other. Except for EGA Hume, Krichauff and Silverstar, where the total ACG content recovered from Bühler Mill were lower than from Quadrumat Junior ($p<0.001$).

Overall, recoveries were lower for the Bühler mill than Quadrumat Junior mill,– presumably a reflection of the progressive particle size reduction and sieving which gave flour that contained less contamination from the bran and germ tissues that contain the ACGs. Concentration, the critical trait in terms of development of yellow colour in YAN, appeared to show significant differences between varieties despite there being no strong correlation between grain concentration and flour concentration. In terms of variety improvement, selection for grain concentration, a relatively quick method, would not be very useful. Rather selection would have to be on the basis of Bühler flour concentration, a more involved procedure in view of the requirement for a milling step. Before any firm conclusions could be reached, a more detailed study would be required to assess the relative effects of genotype and environment on flour ACG concentration.

There were only relatively minor differences in flour recovery for the Bühler compared with quite large differences for Quadrumat milling, an observation which would need to be confirmed. However these preliminary results suggest that for this trait, Quadrumat milling would not be very useful. Bühler flour ACG concentrations are higher than Quadrumat flour concentrations for Sunco, Sunvale and Ruby. Interestingly, Sunco and Sunvale are Australian Prime Hard wheat cultivars selected for high milling quality and flour yield. Targets for breeding would be high recovery in Bühler flour and high flour concentration.

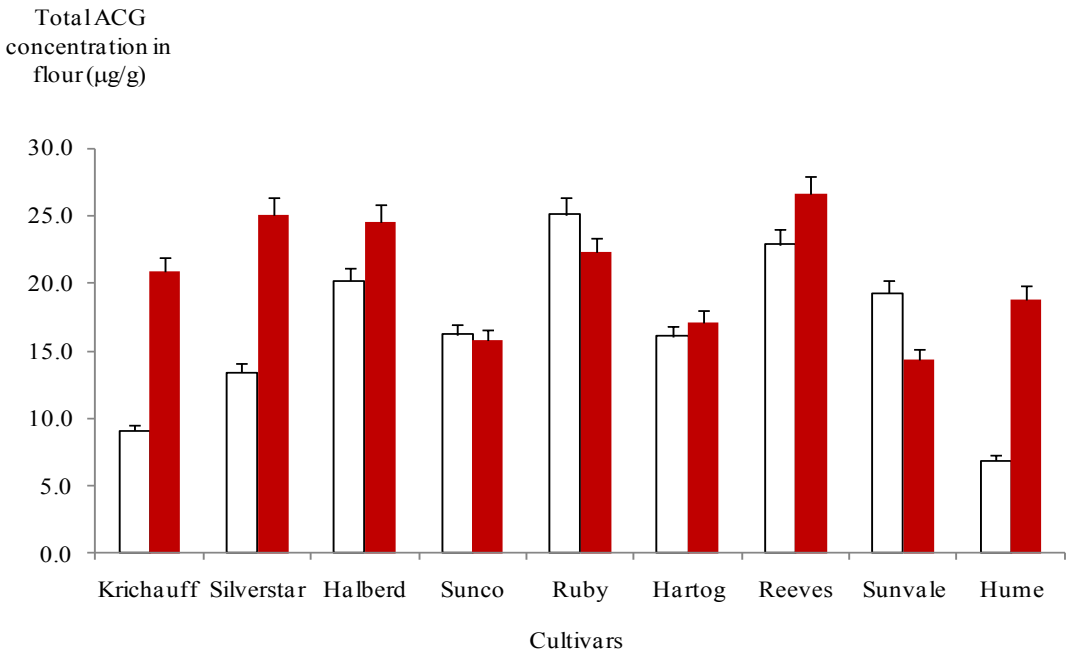

Figure 5.2 ACG concentration (µg/g) in Bühler mill (white bar) and Quadrumat junior mill (red bar) flour of 9 cultivars of bread wheat. The error bars represent the standard errors.

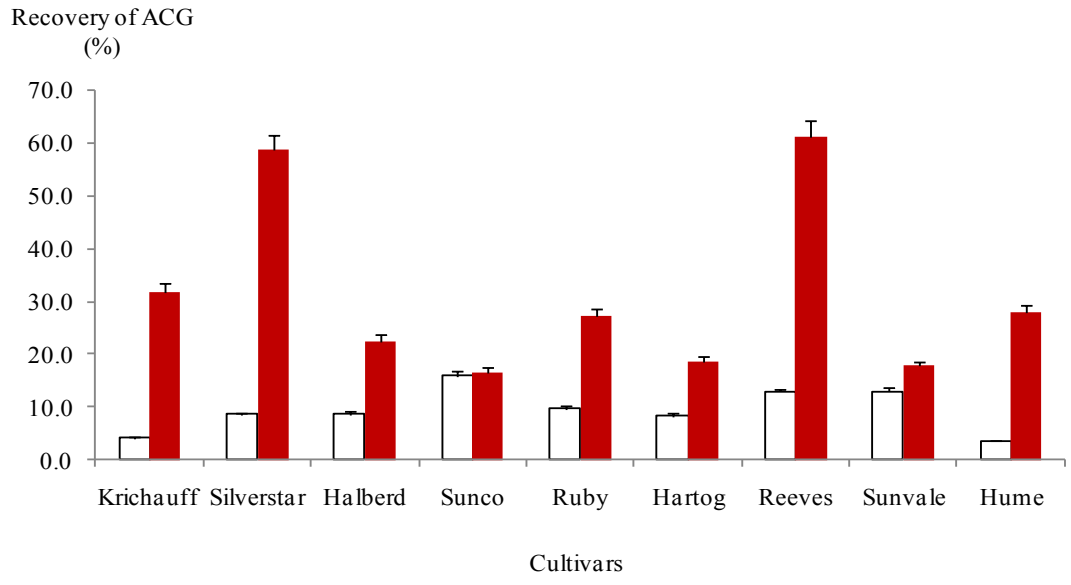

Figure 5.3 Recovery (%) of grain ACG in Bühler mill (white bars) and Quadrumat junior mill (red bars) flours for 9 cultivars of bread wheat. The error bars represent the standard errors.

5.3.1.3 The influence of grain physical properties on milling

The differences in milling recoveries of ACG between varieties may be a reflection of the influence of the grain physical properties on milling. In particular, the softness or hardness of the grain has been shown to influence the milling process (Greffeuille et al. 2005). Grain softness and moisture pre-conditioning influence the efficiency of separation between bran and endosperm in flour milling (Edwards et al. 2007). In maintaining partial dryness for ease of the bran removal, hard grain wheat requires higher moisture than the soft grain to soften the endosperm and toughen the bran (Glenn et al. 1991). In commercial milling practice, soft grain varieties are pre-conditioned to a lower moisture content than hard grain varieties (Edwards et al. 2007). However, in this experiment, all the varieties were hard-grained with the exception of Reeves and the same moisture pre-conditioning was applied to all samples. This might explain the differences in ACG recovery in the flour between the hard grain varieties (Sunco and Sunvale) and the soft grain variety (Reeves), especially when the grains were milled with the less sophisticated Quadrumat Junior mill.

Liyana-Pathirana and Shahidi (2006) reported the higher recovery of phenolic compounds in soft compared with hard grain. Greffeuille et al. (2005) reported that the breaking of wheat grain by milling occurred close to the aleurone layer, at the interphase between tissues, and this determined the differences in biochemical composition of flour recovered from the milling of soft and hard grain varieties. An earlier study by Berliner & Ruter (1928 and 1939), and Mares & Stone (1973) noted that hard wheat grains fractured along the plane of the endosperm walls, while soft grained wheats tend to fracture through the cell itself. However, the difference was not observed with flour recovered from Bühler mill.

There have been intensive studies on the optimization of grain shape, size and hardness for milling, either by adjusting the milling process and recovery (Edwards et al. 2007, Glenn et al. 1991, Greffeuille et al. 2005, Sharma & Anderson 2004) to these grain physical properties or through breeding (Barnes 1986, Barnes & Tester 1987, Bhave & Morris 2008, Gegas et al. 2010, Morris et al. 2005, Morris et al. 2011, Morris et al. 1999, Morrison et al. 1982, Moss et al. 1984, Souza et al. 2002). Various mechanical tools and biochemical markers have been developed to accurately measure the grain physical properties and grain fractures by milling. The ACGs were proposed as biochemical markers for germ contamination of flour, but significant interference from the cinnamic acid derivatives hindered their application in practice (Barnes & Tester 1987, Edwards et al. 2007, Glenn et al. 1991, Greffeuille et al. 2005, Sharma & Anderson 2004). In this study, small number of soft grain and hard grain wheats, with different grain sizes showed significant differences in ACG content and recovery in flour. Further study using a larger and broader germplasm pool with greater variation in grain physical properties is required.

Wheat germ and bran tissues, the by-products of milling, are a source of bioactive compounds such as tocopherols, phytosterols, policosanols, carotenoids, thiamin and riboflavin besides proteins, sugars, fibre, minerals, and lipids that benefit human health (Brandolini & Hidalgo 2012, Morrison et al. 1982, Whent et al. 2012). However, they also contain enzymes that influence noodle colour such as polyphenol oxidase (PPO) and phenolic compounds (Fuerst et al. 2006a, Fuerst et al. 2006b). Incorporation of germ and bran tissue into wheat flour following pre-treatment to stabilize and inactivate the enzymes that influence the end-product quality has been investigated (Ge et al. 2000, 2001). Zhu et al. (2010) reported a positive correlation ($R^2 = 0.9925$) between the

yellowness of flour and the amount of defatted wheat germ mixed with Quadrumat junior mill flour.

5.3.2 Yellowness of YAN and its correlation with lutein and ACG concentration

Yellowness measured at 0 h for YAN made from Quadrumat Junior flour ranged from 19 - 24 b* units (Table 5.3) and there were significant differences ($p<0.001$) between bread wheat cultivars. There was a highly significant correlation between total flour lutein content and YAN b* 0-24 h, and the correlation (r) reduced with time (Table 5.4). No significant correlation between total ACG content and YAN b* were observed.

YAN b* increased with time with measurements at 2, 4 and 24 ranging between 22 -29 (Table 5.3). Significant differences ($p<0.001$) between varieties were maintained. The increase of b* were significant between 0-2 hours, for all varieties. The increase of b* between 2-24 hours were not significant for most of the varieties (17 of them). Nine of the varieties has significantly increased b* between 4-24 hours whilst only 1 variety, Chara, showed a decrease in b* after 2 hours. The increase in b* between 0-2 hours coincided with a significant decrease in L* (supplemental material 5.4). This is most likely a reflection of the negative relationship between L* and b*. The loss of brightness, or reduction in L*, that occurs in the first few hours after preparation of YAN has been shown to be associated with rearrangement within the protein matrix that reduces reflectance rather than any significant chemical change (Asenstorfer *et al.* 2010) whilst Mares et al. (2001) reported that both lutein and ACG were stable in YAN. As a confounding effect, dark pigments produced later during storage of YAN

(Asenstorfer et al. 2010) may mask the yellow pigments, and thereby reduce b* (Mares et al. 2001).

5.3.3 Yellow colour developed in the presence of alkaline salts

The difference in yellow colour between YAN and WSN, Δb* (YAN b*-WSN b*) is associated with compounds such as the ACGs that turn yellow at the higher pH produced by the addition of alkaline salt solution. On the other hand, other compounds such as lutein are yellow in the flour and their yellowness is not pH dependent (Wang 2001). Δb* ranged between 3.9-7.1 at 0 h, 3-7 at 2 h, 2.7-7.8 at 4 h, and 4.3-8.3 at 24 h (Table 5.3) and there appeared to be significant differences ($p<0.001$) between cultivars. Significant differences between varieties ($p<0.001$) in Δb* values were also obtained for measurement of noodle sheets at these 4 different times. The trends in the change of Δb* values between 0 and 24 h were also significantly different between varieties ($p<0.001$) (Fig. 5.4).

Table 5.3 Yellow colour (b*) of YAN and WSN made from Quadrumat Junior Mill flour and the portion of YAN b* and Δb*(YAN b*-WSN b*) developed in the presence of alkaline salt, for 28 bread wheat cultivars at 0, 2, 4 and 24 hour.

Cultivars	YAN b*				WSN b*				Δb*(YAN b*-WSN b*)			
	0	2	4	24	0	2	4	24	0	2	4	24
Ventura	20.1 ± 0.2	22.7 ± 0.1	23.4 ± 0.1	25.5 ± 0.7	14.0 ± 0.1	17.6 ± 0.1	18.0 ± 0.2	18.0 ± 0.1	6.1 ± 0.3	5.1 ± 0.1	5.4 ± 0.3	7.6 ± 0.7
Sunvale	19.9 ± 0.2	22.4 ± 0.1	22.6 ± 0.2	22.5 ± 0.2	14.1 ± 0.2	16.7 ± 0.1	16.5 ± 0.1	14.4 ± 0.2	5.8 ± 0.1	5.7 ± 0.1	6.1 ± 0.2	8.1 ± 0.3
Sunco	20.4 ± 0.3	23.8 ± 0.3	24.5 ± 0.2	25.3 ± 0.1	14.1 ± 0.2	17.1 ± 0.1	17.1 ± 0.2	17.0 ± 0.2	6.3 ± 0.3	6.7 ± 0.2	7.4 ± 0.2	8.3 ± 0.3
Diamondbird	21.3 ± 0.2	23.7 ± 0.1	23.9 ± 0.1	24.2 ± 0.2	15.1 ± 0.1	18.5 ± 0.1	19.1 ± 0.1	18.8 ± 0.1	6.2 ± 0.3	5.3 ± 0.2	4.8 ± 0.1	5.4 ± 0.2
Sunlin	20.4 ± 0.2	23.4 ± 0.1	23.8 ± 0.1	24.0 ± 0.2	15.3 ± 0.2	19.0 ± 0.2	19.1 ± 0.2	18.1 ± 0.1	5.1 ± 0.3	4.5 ± 0.3	4.6 ± 0.3	5.9 ± 0.3
Cunningham	21.2 ± 0.1	23.6 ± 0.1	24.0 ± 0.1	23.8 ± 0.1	14.5 ± 0.3	17.9 ± 0.2	18.1 ± 0.1	17.6 ± 0.1	6.7 ± 0.4	5.7 ± 0.3	5.8 ± 0.2	6.3 ± 0.2
EGA Gregory	21.7 ± 0.2	24.8 ± 0.3	25.5 ± 0.3	26.8 ± 0.2	15.6 ± 0.2	19.2 ± 0.1	19.5 ± 0.2	19.8 ± 0.1	6.1 ± 0.3	5.7 ± 0.3	6.1 ± 0.5	7.0 ± 0.3
Hartog	21.1 ± 0.1	23.7 ± 0.2	24.0 ± 0.2	24.0 ± 0.2	14.4 ± 0.2	18.1 ± 0.1	18.6 ± 0.1	18.1 ± 0.1	6.7 ± 0.2	5.6 ± 0.2	5.4 ± 0.2	5.9 ± 0.2
Janz	20.9 ± 0.2	23.1 ± 0.2	23.5 ± 0.2	23.3 ± 0.2	14.8 ± 0.1	17.7 ± 0.1	17.9 ± 0.2	17.5 ± 0.1	6.1 ± 0.2	5.5 ± 0.2	5.5 ± 0.3	5.8 ± 0.3
EGA Hume	21.2 ± 0.3	23.6 ± 0.1	24.1 ± 0.2	24.5 ± 0.2	15.0 ± 0.1	18.1 ± 0.1	18.3 ± 0.1	18.3 ± 0.2	6.1 ± 0.3	5.5 ± 0.2	5.8 ± 0.1	6.2 ± 0.3
Kennedy	21.5 ± 0.3	24.0 ± 0.1	24.4 ± 0.1	24.1 ± 0.1	14.4 ± 0.1	17.9 ± 0.1	18.2 ± 0.1	18.1 ± 0.2	7.1 ± 0.3	6.1 ± 0.1	6.2 ± 0.1	6.1 ± 0.2
Lang	20.2 ± 0.2	24.1 ± 0.3	25.0 ± 0.3	25.1 ± 0.6	14.1 ± 0.2	17.0 ± 0.2	17.1 ± 0.2	17.2 ± 0.2	6.2 ± 0.4	7.0 ± 0.4	7.8 ± 0.5	7.9 ± 0.6
Batavia	22.3 ± 0.3	25.5 ± 0.3	25.9 ± 0.2	26.3 ± 0.2	16.7 ± 0.1	20.1 ± 0.1	20.3 ± 0.2	20.4 ± 0.3	5.5 ± 0.2	5.4 ± 0.4	5.6 ± 0.4	5.9 ± 0.4
Baxter	20.2 ± 0.2	22.7 ± 0.1	22.9 ± 0.1	23.0 ± 0.2	13.9 ± 0.1	17.4 ± 0.2	17.2 ± 0.2	15.5 ± 0.2	6.2 ± 0.2	5.3 ± 0.2	5.7 ± 0.1	7.5 ± 0.2
Spear	21.7 ± 0.2	24.1 ± 0.1	24.2 ± 0.2	24.7 ± 0.4	15.5 ± 0.2	18.3 ± 0.2	18.4 ± 0.3	17.9 ± 0.2	6.2 ± 0.4	5.8 ± 0.3	5.8 ± 0.4	6.8 ± 0.6
Frame	20.8 ± 0.4	24.2 ± 0.2	25.0 ± 0.2	26.1 ± 0.3	15.7 ± 0.2	19.7 ± 0.1	19.7 ± 0.1	19.1 ± 0.2	5.1 ± 0.5	4.6 ± 0.3	5.3 ± 0.2	7.1 ± 0.3
Halberd	21.1 ± 0.1	23.7 ± 0.1	24.3 ± 0.1	25.1 ± 0.2	16.2 ± 0.1	18.7 ± 0.1	19.2 ± 0.2	18.3 ± 0.1	4.9 ± 0.1	5.0 ± 0.2	5.1 ± 0.2	6.8 ± 0.3
Krichauff	23.5 ± 0.5	27.0 ± 0.4	27.8 ± 0.4	29.3 ± 0.3	19.6 ± 0.2	23.2 ± 0.4	24.5 ± 1.0	22.5 ± 0.2	3.9 ± 0.4	3.9 ± 0.2	3.4 ± 0.9	6.8 ± 0.3
Kukri	20.2 ± 0.2	23.1 ± 0.2	23.8 ± 0.2	25.4 ± 0.5	16.0 ± 0.2	19.3 ± 0.0	19.3 ± 0.1	18.3 ± 0.1	4.2 ± 0.3	3.8 ± 0.2	4.5 ± 0.3	7.2 ± 0.5
Chara	21.3 ± 0.2	23.8 ± 0.2	23.8 ± 0.2	22.9 ± 0.2	16.3 ± 0.3	19.7 ± 0.1	19.5 ± 0.1	18.7 ± 0.1	5.0 ± 0.4	4.2 ± 0.2	4.3 ± 0.1	4.3 ± 0.2
Meering	21.5 ± 0.3	24.6 ± 0.3	24.8 ± 0.3	24.3 ± 0.2	16.5 ± 0.2	19.6 ± 0.2	19.8 ± 0.1	18.9 ± 0.1	5.1 ± 0.3	5.0 ± 0.4	5.0 ± 0.4	5.4 ± 0.2
Silverstar	21.4 ± 0.3	24.5 ± 0.2	24.8 ± 0.1	24.4 ± 0.2	15.5 ± 0.7	17.8 ± 0.1	18.0 ± 0.1	17.5 ± 0.2	5.9 ± 0.5	6.7 ± 0.2	6.9 ± 0.2	6.9 ± 0.3
Annuello	21.7 ± 0.2	25.0 ± 0.1	25.1 ± 0.1	24.7 ± 0.1	15.6 ± 0.1	18.8 ± 0.1	19.0 ± 0.2	18.1 ± 0.2	6.1 ± 0.2	6.2 ± 0.2	6.1 ± 0.1	6.7 ± 0.2
GBA Ruby	19.7 ± 0.1	22.6 ± 0.2	22.9 ± 0.1	23.3 ± 0.1	13.5 ± 0.2	16.4 ± 0.1	16.6 ± 0.1	16.5 ± 0.1	6.2 ± 0.2	6.3 ± 0.2	6.4 ± 0.2	6.7 ± 0.2
Cascades	22.5 ± 0.2	24.9 ± 0.3	25.1 ± 0.2	24.3 ± 0.2	18.2 ± 0.3	21.1 ± 0.2	21.1 ± 0.1	18.7 ± 0.5	4.3 ± 0.3	3.9 ± 0.2	4.0 ± 0.1	5.6 ± 0.5
Carnamah	21.8 ± 0.4	23.3 ± 0.2	23.1 ± 0.2	22.8 ± 0.3	17.7 ± 0.3	20.3 ± 0.2	20.4 ± 0.3	17.8 ± 0.3	4.1 ± 0.4	3.0 ± 0.3	2.7 ± 0.4	5.1 ± 0.5
Reeves	20.5 ± 0.2	23.6 ± 0.1	23.8 ± 0.2	24.1 ± 0.1	14.5 ± 0.2	17.9 ± 0.1	18.2 ± 0.1	19.3 ± 0.1	6.0 ± 0.1	5.8 ± 0.2	5.6 ± 0.2	4.7 ± 0.2
Westonia	23.0 ± 0.3	25.6 ± 0.1	25.7 ± 0.1	25.9 ± 0.2	17.6 ± 0.2	20.9 ± 0.2	21.4 ± 0.1	20.2 ± 0.1	5.4 ± 0.2	4.6 ± 0.1	4.4 ± 0.2	5.6 ± 0.2
Max	23.5	27.0	27.8	29.3	19.6	23.2	24.5	24.0	7.1	7.0	7.8	8.3
Min	19.7	22.4	22.6	22.5	13.5	16.4	16.5	14.4	3.9	3.0	2.7	4.3
Means	21.2 ± 0.2	24.0 ± 0.2	24.3 ± 0.2	24.6 ± 0.3	15.5 ± 0.3	18.7 ± 0.3	18.9 ± 0.3	18.2 ± 0.3	5.7 ± 0.16	5.3 ± 0.2	5.4 ± 0.2	6.4 ± 0.2

Table 5.4 Correlation between total lutein content and ACG content in flour with YAN b* and Δb* (YAN b*-WSN b*). # signifies the significant correlations.

Yellowness (b* and Δb*)	Time of measurement	Total lutein	Total ACG
b* of YAN	0 hours	#0.821	0.126
	2 hours	#0.749	0.106
	4 hours	#0.666	0.032
	24 hours	#0.497	-0.110
Δb* (b*YAN-b*WSN)	0 hours	# -0.731	-0.454
	2 hours	#-0.675	-0.224
	4 hours	#-0.724	-0.266
	24 hours	-0.380	-0.342

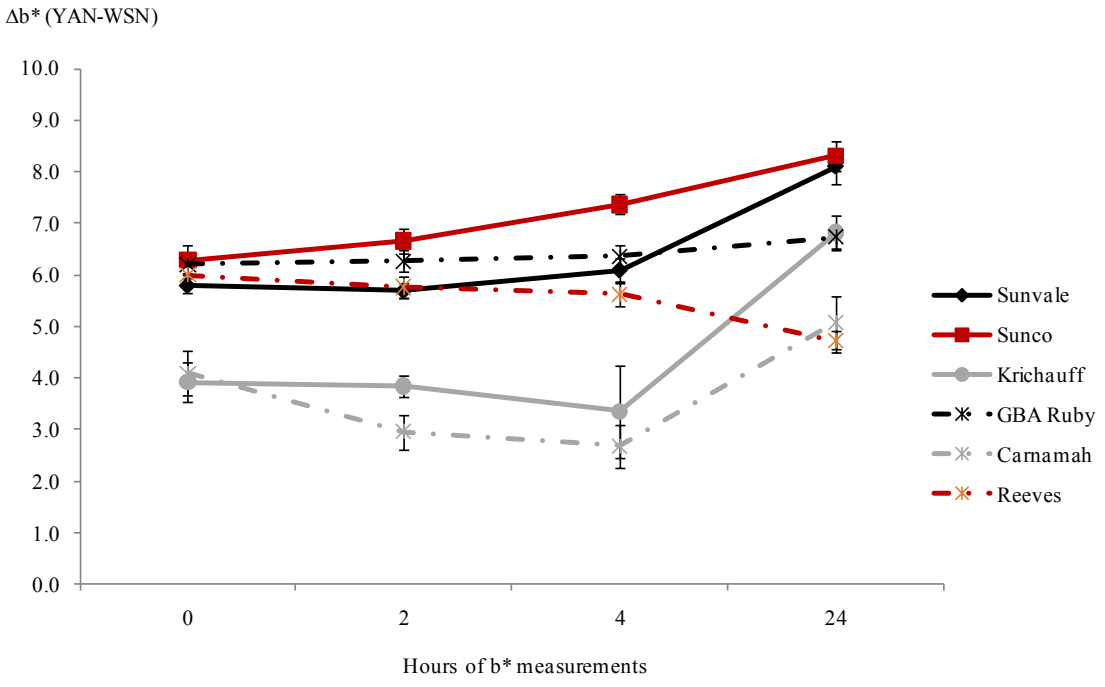

Figure 5.4 The trends of Δb* values between 0 to 24 hours measurements for varieties that showed significant differences ($p<0.001$).

Flour ACG content and YAN Δb* at 0, 2 or 24 hours were not significantly correlated (Table 5.3). Although it seems clear that ACGs can contribute to the yellow colour of alkaline noodles (Asenstorfer et al. 2006), ACG content did not influence the differences in intensity of the yellow colour between varieties. This may be a reflection of the relatively narrow range in flour ACG content, 11-28 μg/g (Tables 5.1 and 5.3) compared with the difference in ACG content required to produce a significant change in b*. It would appear that cultivars with wider range of ACG content in the flour and Δb* value would be required to make a significant impact on YAN Δb*. From the survey of genetic variation in ACG content and composition (Chapter IV), the range of total ACG content in whole grain is 42.8 to 166.6 μg/g (Table 4.1). Similarly, in this experiment, total grain ACG content ranged from 42.8-109.2 μg/g but only 11.4-28.4 μg/g was recovered in the flour (Table 5.1). The lack of correlation between the ACG content in the grain and in the flour suggests that the amount of ACG in the flour is not determined by the content in the grain, but by milling.

The strong negative correlation between lutein content and Δb* at 0-4 h, but not at 24 h, is difficult to explain since there is no evidence in the literature that change in pH affects the absorption of lutein in the visible region of the spectrum. It is possible that this result is an artifact due to the specific characteristics of the varieties selected for this study as representing the range in ACG and lutein content. In particular, a closer examination of the results indicated that the correlation was largely due to high lutein varieties such as Krichauff developed in southern Australia and not selected for YAN quality. In comparison, there was no significant correlation between lutein concentration and Δb* for a subset of varieties developed in northern NSW and Queensland where there is a strong focus on YAN.The lack of positive correlation

between the increase of Δb* from 0 to 24 hours and the pigment content suggests that lutein and ACGs contribute to yellow colour of noodles at 0 hour, but that the increase in b* with time after alkaline solution addition has nothing to do with these two yellow pigments but are possibly influenced by a complex interaction involving the time-dependent darkening of noodles. The initial darkening (Asenstorfer et al. 2010), involves a physical change in the noodle sheet that results in a reduction of reflectance. Beyond 2-4 h, production of dark pigments (from both PPO and non-PPO reactions) occurs, which have the effect of masking yellow pigments (supplemental material 5.4) (Mares et al. 2001). The noodles appear darker and more yellow. The amount of darkening due to these reactions varies with both variety and environment. Both lutein and ACG are stable in YAN (Mares et al. 2001), the yellow colour is already present, lutein, or generated immediately upon the addition of alkali, ACG, and there appears to be no reason to suggest that the colour yield of either should change with time. As a result the data obtained at 0 h is likely to be the best estimate of the specific contribution of these pigments to the colour of noodles and the data collected at 2-24h is likely to be confounded by other factors.

5.3.4 Prediction of the contribution of ACG to Δb (YAN-WSN)*

In the absence of a supply of authentic ACG, a standard curve was constructed by adding known amounts of rutin, an apigenin-*C*-glycoside with the same chromophore as ACG, to flour prior to the preparation of YAN (Fig. 5.2). With increasing amount of rutin, Δb*(YAN with rutin – YAN control) initially increased linearly but then the increase in Δb* became progressively less per unit increase in rutin. Curves for Δb*(YAN with rutin – YAN control) determined at 0, 2 and 4 h were similar and significantly greater than for measurements taken at 24h. By using Δb* from YAN with

rutin – YAN control, little effect of darkening would be expected, since it should be similar in YAN with rutin and YAN control, unless rutin itself is contributing to the darkening.

Based on the standard curve, the predicted contribution of flour ACG concentrations of 11.4-28.4 μg/g to YAN b* would be 1.0-3.0 and 0.8-1.7 respectively depending on whether the curves at 0-4 h or 24 h are used. By comparison, if all the ACG present in grain (Chapter IV), 42.8 to 166.6 μg/g, could be recovered in flour the contribution to YAN b* would be 3.5-11.9 for 0 to 4 h and 2.4-5.4 for 24 h. The ACG concentration in the grains of cultivars used in the Quadrumat milling experiments ranged from 42.8-109.2 μg/g, would be equivalent to increases in YAN b* of 3.5-9.0. There is potential to increase the Δb* value by selecting cultivars with higher ACG content in the grain and by also considering recovery during milling. There is also potential perhaps to achieve the increase in Δb* of YAN by mixing stabilized germ back into flour after milling. Further study is required to explore this possibility.

5.3.5 Contribution of ACG to Δb(YAN –WSN)*

The predicted contribution of ACG to YAN b*, mean at 0, 2 or 4 h of 1.6 – 2.1 (Table 5.5), was significantly less than the observed values of Δb* (YAN-WSN), 5.3 – 5.7 (Table 5.3). This difference between the predicted and actual measurements indicates the presence of other factors that influence the yellow colour of alkaline noodles.

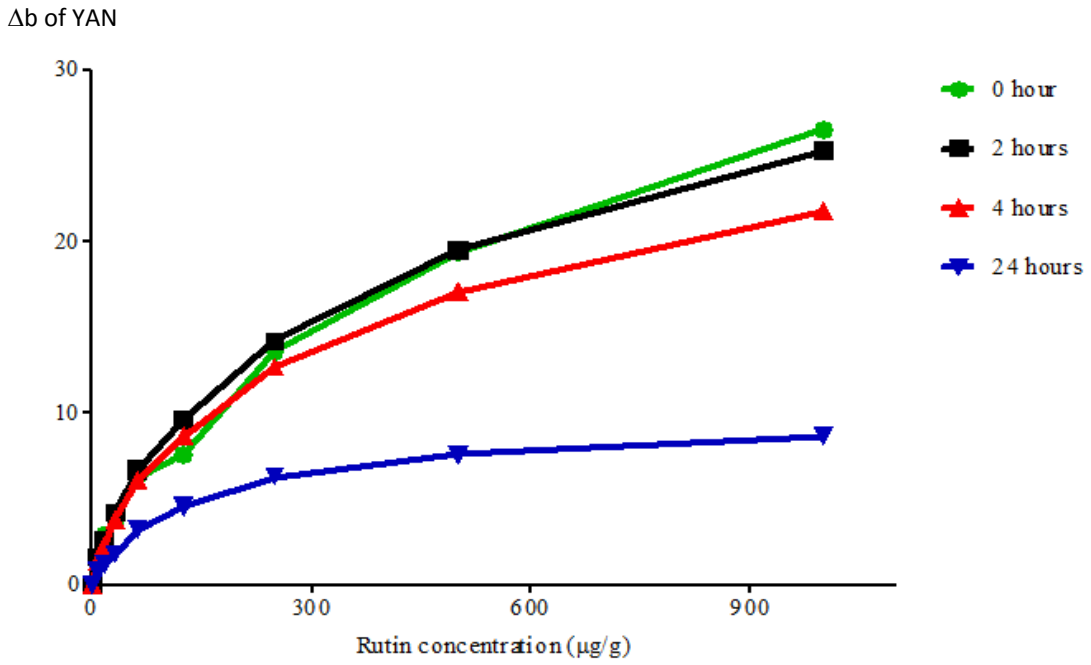

Figure 5.5 Standard curve relating change in YAN b* (Δb* = b*YAN with rutin – b*YAN control) to the amount of added rutin (a flavone glycoside) at 0, 2, 4, and 24. Green: Δb* at 0 hours, black: 2 hours, red: 4 hours, and blue: 24 hours.

Table 5.5 The predicted values for Δb* of YAN sheets made from grain of 28 cultivars of bread wheat milled by Quadrumat Junior Mill, based on rutin-Δb* standard curve (Figure 5.2).

Cultivars	Total ACG (μg/g)	Δb* of YAN			
		0 hours	2 hours	4 hours	24 hours
		36.4x/(406.4 + x)	31.2x/(271.5 + x)	26.2x/(241.3 + x)	9.7x/(133.7 + x)
Ventura	15.5	1.3	1.7	1.6	1.0
Sunvale	14.4	1.2	1.6	1.5	0.9
Sunco	15.8	1.4	1.7	1.6	1.0
Diamondbird	11.4	1.0	1.3	1.2	0.8
Sunlin	17.3	1.5	1.9	1.8	1.1
Cunningham	18.0	1.5	1.9	1.8	1.2
EGA Gregory	13.3	1.1	1.5	1.4	0.9
Hartog	17.1	1.5	1.9	1.7	1.1
Janz	20.0	1.7	2.1	2.0	1.3
EGA Hume	18.8	1.6	2.0	1.9	1.2
Kennedy	16.8	1.4	1.8	1.7	1.1
Lang	19.1	1.6	2.0	1.9	1.2
Batavia	14.8	1.3	1.6	1.5	1.0
Baxter	16.1	1.4	1.7	1.6	1.0
Spear	16.9	1.5	1.8	1.7	1.1
Frame	18.8	1.6	2.0	1.9	1.2
Halberd	24.6	2.1	2.6	2.4	1.5
Krichauff	20.8	1.8	2.2	2.1	1.3
Kukri	21.0	1.8	2.2	2.1	1.3
Chara	18.4	1.6	2.0	1.9	1.2
Meering	28.4	2.4	3.0	2.8	1.7
Silverstar	25.1	2.1	2.6	2.5	1.5
Annuello	21.4	1.8	2.3	2.1	1.3
GBA Ruby	22.2	1.9	2.4	2.2	1.4
Cascades	20.9	1.8	2.2	2.1	1.3
Carnamah	27.4	2.3	2.9	2.7	1.7
Reeves	26.6	2.2	2.8	2.6	1.6
Westonia	20.0	1.7	2.1	2.0	1.3
Max	28.4	2.4	3.0	2.8	1.7
Min	11.4	1.0	1.3	1.2	0.8
Means	19.3	1.6	2.1	1.9	1.2

5.4 Conclusions

ACGs make a small contribution to the yellow colour of alkaline noodles (YAN), but explain only a part of the yellow colour that develops specifically in the presence of alkaline salts. Possibly due to a combination of this small contribution, the limited variation in ACG concentration between cultivars, and the relatively limited variation in Δb*(YAN – WSN), no correlation between flour ACG concentration and YAN b* or

Δb* was observed. Unfortunately much of the grain ACG is discarded during the milling process and there appears to be little prospect of significantly increasing the proportion of grain ACG recovered in flour.

Given the low contribution of ACGs to YAN yellowness, this compound might not be a target that would be of interest to the breeders. However, in this study, further possibilities and efforts to increase the ACG recovery in the flour and their contribution to the yellowness of YAN were explored, further examined and discussed.

Bühler milling improved flour recovery and the amount of ACG recovered in the flour for 3 out of 10 varieties, but these improvements were not significant and not enough to achieve the yellowness of commercial noodles containing additives (b*= $\pm$ 45). It would required greater than 500 mg/g ACG to achieve this b* value. In addition, the percentage of ACG recovered in the Bühler flour is generally less to that of Quadrumat milling. Based on this observation it appears unlikely that recovery of ACG in flour in a commercial mill would be significantly different. The recovery of ACG in mill flour is strongly influenced by their location in the outer parts of the grain (grain coat and embryo tissues) which are discarded during milling as bran and pollard respectively. The efficiency of removal of these parts from the starchy endosperm during milling is influenced by the grain softness/hardness and moisture pre-conditioning.

Cultivars with substantially higher grain ACG concentration that give a much higher recovery in flour are required for improvement of YAN b*. The ACG content in wheat grain still showed potential for improvement of ACG concentration in flour but the

physical properties of the grain need to be improved for a better milling recovery. Further study involving germplasm with wider variation in the grain physical properties is required in order to relate these physical and the ACG traits. Moreover, as there was variation in ACG recovery in the flour after grain milling, further study is required to explore this potential in relation to the possibility to achieve the increase in Δb* of YAN by mixing defatted or stabilized wheat germ back into flour after milling.

The results in this experiment also indicated the presence of unknown pigment compound(s) in addition to ACGs that contribute to the yellowness of YAN. Further research is required to determine the structure and tissue location of these compounds in relation to their recovery from grain milling.

In comparison with lutein, the ACGs are present in higher concentration in grain and flour but their contribution to yellowness of YAN is much lower than lutein. Due to its presence in starchy endosperm, an increase in lutein concentration could deliver substantially higher YAN b*. In the current market environment this may be unacceptable since the yellow flour would be undesirable in products other than YAN.

Title of Thesis: Genetic control of Apigenin di-*C*-glycosides biosynthesis in bread wheat (*Triticum aestivum* L.) grain and their potential as yellow pigment of Asian alkaline noodles

Student Name: Grace Yasmein Wijaya

Pg # PhD

Chapter VI

QTL associated with Apigenin di-C-glycosides (ACG) content and composition in bread wheat (*Triticum aestivum* L.) grain and the identification of candidate genes

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STATEMENT OF AUTHORSHIP

Title of Paper QTL associated with Apigenin di-C-glycosides (ACG) content and composition in bread wheat (*Triticum aestivum* L.) grain and the identification of candidate genes

Text in manuscript

Name of candidate Grace Yasmein Wijaya

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Designed and developed the detail experiment for data collection, performed the biochemistry analysis on all samples (excluded HPLC machine set-up), marker analyses (SSR and SNP) and bioinformatic data exploration and analysis, analyzed and interpreted the data, developed the illustration used in the manuscript, planned the article, wrote manuscript and acted as corresponding author.

Certification that the statement of contribution is accurate

Signed ....*Date*..19 March 2012

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Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

External advisor of the project; provided resources for marker analysis (background IP for the methods, equipment, chemicals, softwares), provided initial information and data on Sunco x Tasman population (genotyping), provided critical evaluation, supervised development of work and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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co-author name Prof. Mrinal Brave and Huimei Wu

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

External collaborator of the project, provided the *CypB* genotyping data, and provided manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed ..... Date 26/3/12

Signed ..... Date 27/3/12

co-author name Dr. Amanda R. Walker

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

External supervisor of the project; provided critical evaluation, suggestions and advised in the exploration of the bioinformatic data and sequence analysis, supervised development of work and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed ... Date 20/3/2012

co-author name Assoc. Prof. Daryl Mares

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Principal supervisor of the project, developed the initial outline for the project, provided background IP for the project, all the germplasm and genetic stocks requested, provided critical evaluation, supervised development of work and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed ... Date 20/3/2012

Chapter VI: QTL associated with Apigenin di-*C*-glycoside (ACG) concentration and composition in bread wheat (*Triticum aestivum* L.) grain and identification of candidate genes

6.1 Introduction

Glycosylation reactions like those responsible for the synthesis of apigenin di-C-glycosides (ACG) have important roles in modification and diversification of metabolites that are involved in various functions from structure and storage to signalling in plants (Breton et al. 2006). However, despite their diverse and important roles the genetic control of the ACG biosynthesis pathway, a side branch in the flavonoid pathway, in cereals is not well understood.

Following conversion of naringenin chalcone to 2S-naringenin flavanone by chalcone isomerase (CHI), the first committed step towards ACG synthesis appears to involve the formation of the hemiketal form of the flavanone by flavanone 2-hydroxylase (F2H). The hemiketal ring structure then opens prior to *C*-glycosylations which lead to the formation of 2 sets of Wesseley-Moser isomers containing arabinose and glucose (ACG1) or arabinose and galactose (ACG2). The reactions that follow the *C*-glycosylation are similar for both ACG1 and ACG2. Chalcone and flavanone are also precursors for the biosynthesis of other metabolite in flavonoid biosynthesis (Grotewold et al. 1998, Shirley 2001).

In the earlier part of this study, a survey of 67 bread wheat cultivars and related species confirmed the presence of genetic variation in ACG concentration and composition as previously reported by Asenstorfer et al. (2006). The results for the CS nullisomic-tetrasomic together with the frequency distribution of ACG content and ratio traits in the set of varieties and related species suggested that concentration and ACG1/ACG2 ratio may be controlled by multiple loci and a single locus respectively. It is not clear whether the synthesis of ACG1 and ACG2 involves a glycosyltransferase that is capable of utilizing both UDP-glucose and UDP-galactose or glycosyltransferases that are specific to each of these sugar nucleotides. Both *C*-glucosylation and *C*-galactosylation reactions contribute in a coordinated manner to the total concentration and ratio of ACGs. However, although there was more ACG2 than ACG1 in the grain of most of the cultivars surveyed, variation in the ratio appeared to be due to an alteration in *C*-glucosylation. Further molecular and enzymatic studies are required to investigate the variation in availability of the sugar molecules and *C*-glycosyltransferase activity.

Only limited information is available on the biochemistry and molecular biology of *C*-glycosylation from previous work. Brazier-Hicks et al. (2009) reported a *C*-glucosyltransferase gene specific for UDP-glucose in rice. Du et al. (2010) and Du et al. (2009) reported that *F2H* genes in rice and sorghum code for the enzyme that provides the substrate for the *C*-glucosylation reaction. However, no information is available on such genes in wheat. In addition, there are two other enzymes that have been studied in wheat and other crops, UDP-glucose 4-epimerase (Zhang et al. 2006) and a UDP-glucose transporter (Bowsher et al. 2007, Muaoz et al. 1996), which might also be involved in providing sugar molecules for the *C*-glycosylation reaction. It should be

noted here that these sugar nucleotides are also required for several other pathways, and no information on their contribution to *C*-glycosylation has been reported.

Gene discovery in hexaploid bread wheat has been facilitated by genetic mapping. This approach involves generating the genetic linkage map of the experimental populations using RFLP, AFLP, SSR and SNP markers, or takes advantage of their existing genetic linkage maps. Preliminary QTL analysis detects the regions for fine mapping that is required to capture candidate gene sequences from syntenic regions of chromosomes in other grass species or from BAC clones. The availability of nullisomic-tetrasomic, ditelosomic and substitution lines of Chinese Spring aid this approach if the trait of marker is present in the Chinese Spring euploid, to confirm the chromosome location of the traits or new markers.

The aims of this study were to validate QTL associated with variation for ACG concentration and ACG1/ACG2 ratio in bread wheat grain, to add more markers to the QTL region and reduce the size of the chromosome interval involved, and to identify candidate gene(s) linked with these traits. It is anticipated that this information will provide further insight into the mechanisms involved in ACG biosynthesis in bread wheat and provide molecular tools required for manipulation of these traits in breeding programs.

6.2 Material and methods

6.2.1 Plant material

A sub-set of a Sunco x Tasman doubled haploid population developed by (Kammholz et al. 2001) consisting of 153 lines and 361 markers (section 3.2.3.2) was used for the

initial mapping and QTL analysis. Subsequently, a larger set of 275 doubled haploid lines was used for the fine mapping. For the preliminary polymorphism screening of new SSR markers, a panel of Sunco/Tasman doubled haploid lines (10 lines with ratio phenotype similar to Sunco (high ratio) and 10 similar to Tasman (low ratio) (section 3.2.3.3) were used. The group 7 nullisomic-tetrasomic lines and ditelosomic lines of Chinese Spring 7 (section 3.2.2 and section 3.2.3.2) were used to confirm the chromosome arm locations and to get genome sequences for designing SNP markers (section 6.2.3.3 SNP marker analysis).

6.2.2 Validation of the QTL associated with variation in ACG composition, ACG concentration, and 100 grain weight in the Sunco/Tasman

6.2.2.1 Construction of new genetic maps for Sunco/Tasman incorporating additional markers

The genetic map of the 153 lines Sunco/Tasman doubled haploid population was re-constructed with 361 markers using Mapdisto 1.7.5. b4 2007 version and GENSTAT (version 14 (Chapter 3 section 3.4.2.2). Markers were ordered within each linkage group and distances between markers computed using Mapdisto 1.7.5. b4 2007 version. The distances in the map were then confirmed with GENSTAT 14) before the QTL analyses were performed. Using GENSTAT software, the linkage groups were re-constructed with a 0.365 recombination factor and optimising the clustering with 0.5 penalty for each missing data point. The results were saved in a QTL save structure and then exported and viewed in Flapjack (Plant Bioinformatic Group, The James Hutton Institute) to check for errors and double recombination. The complete set of

chromosome maps was drawn using MapChart 2.2 (Plant Research International, Wageningen Agricultural University, The Netherlands).

6.2.2.2 QTL analyses of ACG concentration, ACG composition and 100 grain weight.

The results of an initial QTL analysis of Sunco/Tasman doubled haploid population by Willsmore and Mares (unpublished data) for ACG concentration, total ACG content and ACG ratio using MapManager QTX software were confirmed in the new analysis performed in GENSTAT 14. Details on the data sets used in this analysis are available in the general method section. In addition, QTL analysis for 2 other traits, the ACG1 and ACG2 content were also performed with the same method. The genetic predictors of doubled haploid (DH1) population were calculated according to the Kosambi function, at marker positions (by default) and between markers (every 10 and 20 cM). Single trait linkage analysis (Single environment) was performed for 100 grain weight, ACG concentration and ACG composition. The QTL for ACG1/ACG2 ratio was also analysed as a binary trait (0/1), while the other traits were treated as quantitative traits. Significant thresholds were set up as default (=0.05), based on a Bonferroni correction as described by (Li & Ji 2005). The initial genome-wide scans were performed (Simple Interval Mapping, SIM) to obtain candidate QTL positions. The QTL effects were estimated by Composite Interval Mapping (CIM) using the candidate QTLs detected in the initial scan by SIM as cofactors. The minimum cofactor proximity and minimum separation for selected QTLs were set at 30 cM and 10 cM respectively. REML variance component analyses were performed by fitting the genotype factor as a random model. The fitting of QTL into the fixed model were assessed by Wald statistic and F statistic (Fpr<0.001). The final set of QTL positions and effects were estimated by

fitting the final QTL model following backwards selection from a set of candidate QTLs. The result of Wald statistic or the probability value on a -log10 scale then were plotted to produce the map. The results were saved in a QTL save structure and exported and viewed in Flapjack (Plant Bioinformatic Group, The James Hutton Institute). These steps followed the example outlined in (Malosetti *et al.* 2011).

The effects of QTL detected for each ACG trait were further examined by determining the interaction between the QTL. The lines of Sunco/Tasman doubled haploid population were grouped into marker allele classes according to the genotypes of the closest markers. The analyses were performed by Mixed Models (REML variance components analysis) in GENSTAT 14 with markers and marker interactions as fixed model and the number of lines in each marker genotype group as random model. Epistasis was detected by significant marker interactions at Fpr <0.001.

Multi-trait linkage analysis (single environment) was performed for chromosome 7B for the concentration and content per grain of ACG1 and ACG2 (μg/g and μg/grain); and for the ACG1/ACG2 ratio. Similar analysis was also performed for chromosome 4B for the total ACG (μg/g and μg/grain), ACG1 and ACG2 to obtain a final set of estimated QTL effects.

6.2.3 Addition of markers to the major QTL for ACG concentration and composition located on 7BS

6.2.3.1 DNA Isolation

The DNA was extracted using a mini prep ball bearing DNA extraction protocol (Karakousis & Langridge 2003). Each leaf sample was placed in a screw cap tube together with 1 large (9 mm) and 3 small ball bearings. The tubes then were frozen in liquid nitrogen for 5 minutes and then placed on a shaker for 1 minute to grind the leaf tissue to a fine powder. After the ball bearings were removed, the samples were thawed at room temperature for 2-5 minutes. Then, 700 μL extraction buffer was added to the samples, followed by addition of 700 μL phenol/chloroform/iso-amyl alcohol (25:24:1). After mixing well, the extracts were transferred into silica matrix tubes and spun at 4000 rpm for 10 minutes. A second phenol extraction was done and the upper aqueous phase transferred to eppendorf tubes. The samples were then washed by adding 60 μL of 3M sodium acetate pH 4.8 and 600 μL isopropanol. The tubes were inverted to precipitate the DNA. After centrifugation at 13000 rpm for 5 minutes, the supernatant was poured off and the remaining pellet was washed again with 70% ethanol and then centrifuged for 2 minutes at 13000 rpm. After the ethanol was removed, the samples were air-dried. The pellet obtained was resuspended in 50 μL of RNAse at 4^{o}C overnight and then stored at -20^{o}C.

6.2.3.2 SSR marker analysis

Polymorphism test

DNA samples from a panel of 20 Sunco/Tasman lines (section 3.2.3) and 2 DNA samples of each of the parents were used for the initial SSR marker polymorphism test.

68 SSR markers that had previously been mapped on 7B chromosome between gwm400-wmc76 and wmc76-gwm573 in wheat genetic maps of various populations in CMap databases (Genica, Grain Genes, CYMMYT databases), 7BS1-0.27-1.00 and C7BS1-0.27 bins of deletion map (Sourdille et al. 2004b) and published maps (Boyko et al. 1999, Carter et al. 2009, Crossa et al. 2007, Emebiri et al. 2010, Gupta et al. 2002, Li et al. 2008, Lin et al. 2008, Liu et al. 2005, Panfili et al. 2004, Quarrie et al. 2005, Schnurbusch et al. 2004, Shermana et al. 2010, Song et al. 2005, Sourdille et al. 2003, Sourdille et al. 2004a, Sourdille et al. 2004b, Uphaus et al. 2007, Xue et al. 2008, Zhang et al. 2008) were chosen for this test.

The analysis was performed by multiplex PCR according to (Hayden et al. 2008a, Hayden et al. 2008b). Marker binning information was drawn from Genica database and Automated Designer for Genetic Analysis was used to design the PCR analyses. The 7 μL PCR reaction contained 0.2mM dNTP, 1x Multiplex Ready Buffer, 75 nmol dye labelled tagR and tagF primers (vic, fam, ned, pet), 50ng genomic DNA, 0.175U Immolase DNA polymerase (Kit from Bioline), optimal concentration of locus specific primers as determined in uniplex assays and water. PCR condition was as follows: denaturation at 94^{o}C for 2 minutes, 4 cycles of: 92^{o}C for 30 seconds, 50^{o}C for 1:30 minutes, and 72^{O}C for 1 minute, 19 cycles of: 92^{o}C for 30 seconds, 63^{o}C for 1:30 minutes and 72^{o}C for1 minutes, 39 cycles of: 92^{o}C for 15 sec, 54^{o}C for 1 minute, 72^{o}C for1 min, final extension of 72^{o}C for 10 minutes. Visualization of the PCR products was performed by GelScan2000 (Corbett Research) and ABI3730 DNA analyser (Applied Biosystems). SSR allele sizing and polymorphism analysis were performed automatically using GeneMapper v3.7 software (Applied Biosystems).

Mapping polymorphic markers in the Sunco/Tasman doubled haploid population

Thirty seven polymorphic SSR markers were assayed on each of 275 lines of the Sunco/Tasman population.

6.2.3.3 Single nucleotide polymorphism (SNP) marker analysis

Source of SNPs

Six expressed sequence tags (EST) sequences from chromosome 7B of wheat containing SNPs that are polymorphic in Sunco and Tasman parents and with homologous sequences in rice chromosome 6 and 8 (Sorrells et al. 2003) and *Brachypodium distachyon* chromosome 1 and 3 (ModelCrop database) were used to align the respective maps (Table 6.1). Information on these 6 single nucleotide polymorphisms (SNPs) was obtained from Dr. Matt Hayden, Victorian DPI (pers. com.). Information on the genome specific primer pairs (GSP) flanking these SNPs were drawn from Wheat SNP databases (http://wheat.pw.usda.gov/SNP/new/index.shtml). The lists of wheat EST sequences were drawn from wEST-SQL databases (http://wheat.pw.usda.gov/wEST/).

SNP marker analysis

Genome-specific primer pairs (GSP, Table 6.1) were used to amplify SNP-containing regions from DNA samples representing the panel of Sunco/Tasman doubled haploid lines and from nullisomic-tetrasomic and ditelosomic lines of Chinese Spring. Touchdown PCR conditions were optimized for each pair. The PCR products were then separated on 1% agarose gel stained by SYBR safe and the bands that were present in Sunco and Tasman and absent in nulli 7B lines were then purified from the gel using Illustra Gel Purification Kit and sequenced using the GSPs. The sequences obtained

were analysed by Vector NTI Advance version 11 to identify the SNP and design the assay.

A temperature switch PCR (TSP) method described by Tabone et al. (2009) was used to analyse the polymorphism of the SNP markers in the 275 lines of the Sunco/Tasman doubled haploid population. The amplification was performed using a pair of locus specific primers (LSP) and a pair of allele specific primers (ASP) that were designed as explained by Hayden et al. (2009) and Hayden et al. (2010). Non-complementary nucleotides at 5‟ terminal were included in the LSPs to increase the Tm from 43-47^{o}C (optimum 45^{o}C) to 52-55^{o}C (optimum 53^{o}C) while the ASPs were designed with Tm of 60-65°C (optimal 63°C).

The PCR was performed in a 4 µl reaction containing 1× PCR buffer (16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 100 mM Tris-HCl, and pH 8.3, Bioline), 1.5 mM $MgCl_2$, 100 ng/µl bovine serum albumin, 0.2 mM dNTP, 0.1 µM each of forward and reverse LSP primer, 0.5 µM of forward and reverse ASP primer (unless otherwise stated), 0.15 U Immolase (Bioline) and between 10-50 ng dried down genomic DNA. The PCR condition is as follows: denaturation step at 95°C for 3 minutes, first reaction phase of TSP amplification for a total of 35 cycles with 15 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 60 seconds to enrich the target locus harboring the SNPs, and the second reaction phase consisted of 5 cycles of 95°C for 10 seconds and 45°C for 30 seconds, followed by 15 cycles each consisting of 95°C for 10 seconds, 53°C for 30 seconds and 72°C for 5 seconds. The PCR products were visualized on a 1% agarose gel with SYBR safe staining.

Table 6.1 Wheat EST sequences from chromosome 7B of wheat with homologous sequences in rice (chromosome 6 and 8) and *Brachypodium distachyon* (chromosome 1and 3) and their genome specific primer (GSP) sequences (Wheat SNP database, USDA).

Wheat	Position in Rice (bp)		Position in *Brachypodium distacyon* (bp)		Genome specific primer (GSP)	
EST	Chromosome	Position (bp)	Chromosome	Position (bp)	Forward primer	Reverse primer
BE352570	6	9,092,387-9,092,877 (+)	1	41,582,446-41,582,925 (+)	CAGATCATCCCGTGGAACTT	CGAAACCAGAAACGATGTTG
BE445287	8	25,520,556-25,522,082 (+)	3	42,791,581-42,793,820 (+)	GGGCATGTGTCAAGGGC	TTCCCATGAAGAGGTGGTTC
	8	25,651,324-25,652,211 (+)				
	6	30,377,762-30,378,989 (+)	3	4,026,222-4,026,253 (+)		
BE518436	6	30,377,762-30,378,989 (+)	1	30,748,109-30,750,751 (+)	ATGTCCTTGGAAACTTCCCC	GACCATGTCTAACAGTAAAAC
BF201699	8	24,333,334-24,334,474 (+)	3	41,571,060-41,571,361 (+)	GAAGGGTTTGTGTGGACGAT	TAATCCTTCCAATAATATGATGTAG
		24,463,463-24,464,603 (+)				
BQ169669	8	27,267,082-27,268,137 (+)	3	43,867,506-43,868,726 (+)	GAATTGCAGCCTTAAATGGG	ACTCTTATTTTCATCTCTGG
		27,397,211-27,398,266 (+)				
BQ171683	6	24,579,353-24,579,453 (+)	1	31,562,280-31,563,491 (+)	GTCTCCCAAATTACGAACTTC	GGAGCTTGATGATAACCGGA

6.2.3.4 Apigenin-*C*-diglycoside analysis

ACGs were analysed as previously described in section Chapter 3 section 3.4.1.1 and the values of ACG concentration and composition were calculated as in section Chapter 3 section 3.4.2.1. Hundred grain weight was measured as in section Chapter 3 section 3.4.1.3.

Genetic analysis of ACG trait data was performed using GENSTAT 14. Differences between parent lines were analysis by one way ANOVA with the parent lines as treatment factors. Significant differences were identified at $p<0.05$. The distribution of ACG traits in the 275 lines of the Sunco/Tasman population were tested against Gaussian normal distribution using similar software.

6.2.3.5 Linkage mapping and QTL analysis

The marker scores were analysed for segregation in the population by chi-square analyses (GENSTAT 14). Only 13 new markers that showed no distortion were added to the revised map. These new markers were ordered by MapDisto Genetic Software version 1.7.5 Beta 4 for MS windows 2007 (Mathias Lorieux, Institut de Recherche pour le development, (IRD, France) and International Centre for Tropical Agriculture (CIAT-CGIAR, Colombia).

The QTL analyses for ACG component traits were performed using GENSTAT 14 as mentioned in section 6.2.2.2. The QTL for ACG1/ACG2 was also re-analysed as a binary trait with 0.8 as the critical value; ratio above 0.8 similar to Sunco, while ratio below 0.8 similar to Tasman (Supplemental materials 3.5). This QTL analyses of the ACG traits as binary traits were not performed with other traits because the distributions

of the traits in the populationwere continuous and there was no distinct separation between the high and low value of the traits as there was with the ratio traits.

Comparison of 7BS of Sunco/Tasman with other wheat maps

The Sunco/Tasman genetic map of 7BS was compared with other physical, genetic and consensus maps for wheat in CMap databases (Graingenes (http://wheat.pw.usda.gov/GG2/index.shtml), Genica (http://www.genica.net.au/index.php/GENica), KomugiMap (http://map.lab.nig.ac.jp:8080/cmap/), Gramene (www.gramene.org), CYMMYT (http://cmap.cimmyt.org)). Full sequences of wheat ESTs containing the SNPs were obtained from Wheat Genomic Sequence database (cerealsDB.uk.net), while the ESTs and SNPs information was obtained from Wheat SNP databases (http://wheat.pw.usda.gov/SNP/new/index.shtml). The assembly of wheat contigs were performed by CAP3 (http://pbil.univ-lyon1.fr/cap3.php).

6.2.4 Synteny between wheat, rice and Brachypodium distachyon

6.2.4.1 Wheat 7BS and rice chromosomes 6 and 8

Information on the synteny of wheat 7BS with rice (*Oryza sativa* var japonica) physical and genetic maps was drawn from Wheat Genome Database of JCVI (http://jcvi.org/wheat/wheat_syn_dload.php) and Gramene database (www.gramene.com) respectively. Further detailed information on the BAC and PAC sequences of rice were obtained from Gramene website and their links to NCBI (www.ncbi.nlm.nih.gov/), and ENA of EMBI-EBI (http://www.ebi.ac.uk/ena). Information on the genes in these BAC and PAC sequences was viewed with the Rice

Genome Browser of MSU Rice Genome Annotation (Osa1) Release 6.1 (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/#search).

6.2.4.2 Wheat and *Brachypodium distachyon*

Information on synteny between wheat and *Brachypodium distachyon* was drawn from www.wheatgenome.info and ModelCrop databases. The information from rice, *Brachypodium* and wheat were compiled and ordered based on physical maps in ModelCrop and Gramene databases.

6.2.5 Multiple alignment and structural analysis of candidate sequences

Multiple alignments were performed with MAFFT engine (http://www.ebi.ac.uk/Tools/msa/mafft/). The alignments were then transferred to GeneDoc version 2.7.000 (http://www.nrbsc.org/gfx/genedoc/) for further multiple alignment editing, shading, structure analysis and development of RasMolscript (.scr) files. Initial information on the glycosyltransferases was obtained from the databases of Carbohydrate-Active EnZYmes (www.cazy.org), while information for the protein domain, family and functional sites of the plant *O*-glycosyltransferases were downloaded from Prosite database of ExPASy Swiss Institute of Bioinformatics (SIB) Bioinformatics Resource Portal (http://prosite.expasy.org/). The PDB files for the UDP-glucose: flavonoid 3-*O*-glycosyltransferase from *VvGT1* (2CIX) (Offen *et al.* 2006) were obtained from RSCB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The correlation between the multiple alignments and the structural information of the PDB file were obtained from S2C facility (http://www.fccc.edu/research/labs/dunbrack/s2c). The 3-D molecular structure of the alignment was then viewed by RasWin molecular Graphic version 2.7.4.2

(www.rasmol.org). The amino acid sequence conservation and polymorphism was visualised by weblogo (weblogo.berkerley.edu).

6.3 Results and discussion

6.3.1 ACG content and composition of Sunco/Tasman parents and doubled haploid population

Sunco and Tasman differed from each other for ACG1 and ACG2 concentration, for the ACG1/ACG2 ratio and for ACG2 content per grain, with Sunco having higher ACG1 concentration ($p<0.05$) and ACG1/ACG2 ($p<0.001$) and Tasman having the higher ACG2 concentration and content per grain ($p<0.05$). Although the differences in total ACG concentration, total ACG content per grain and 100 grain weight between Sunco and Tasman were not significant ($p>0.05$), Tasman was slightly higher than Sunco for these three traits. The ACG1 content in the grain of Sunco was higher than Tasman, however, again the difference was not significant ($p>0.05$).

ACG concentration (μg/g) and ACG content (μg/grain) were normally distributed in the population (Figure 6.2) consistent with the results presented in chapter IV and the conclusion that these traits appear to be controlled by multiple genes. By contrast, the frequency distribution for ACG1/ACG2 ratio was bimodal (Supplemental material 3.5). This is also consistent with the results in chapter IV and the conclusion that the observed variation for this trait in the Sunco/Tasman population is regulated by a single gene.

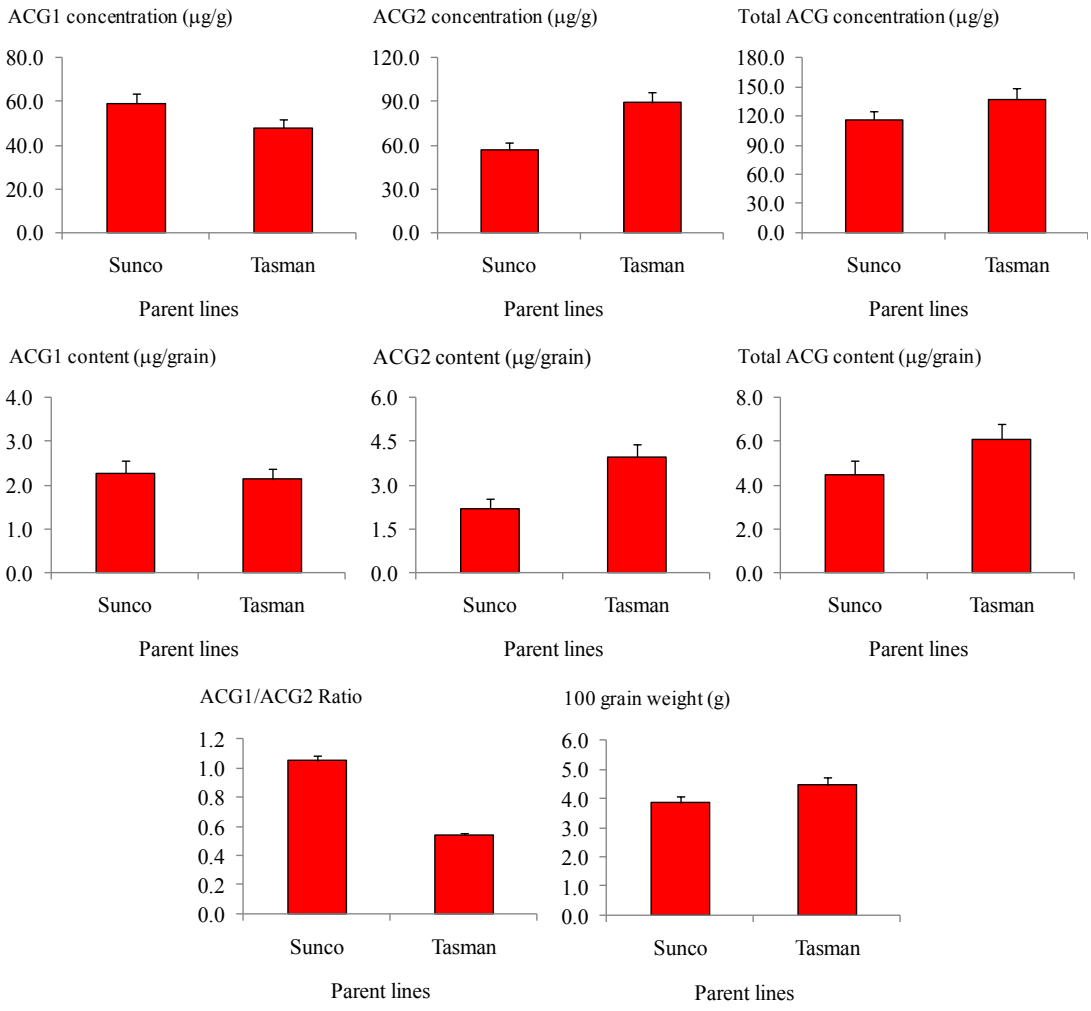


Figure 6.1 The ACG traits in the parents of the Sunco/Tasman doubled haploid population used for fine mapping.

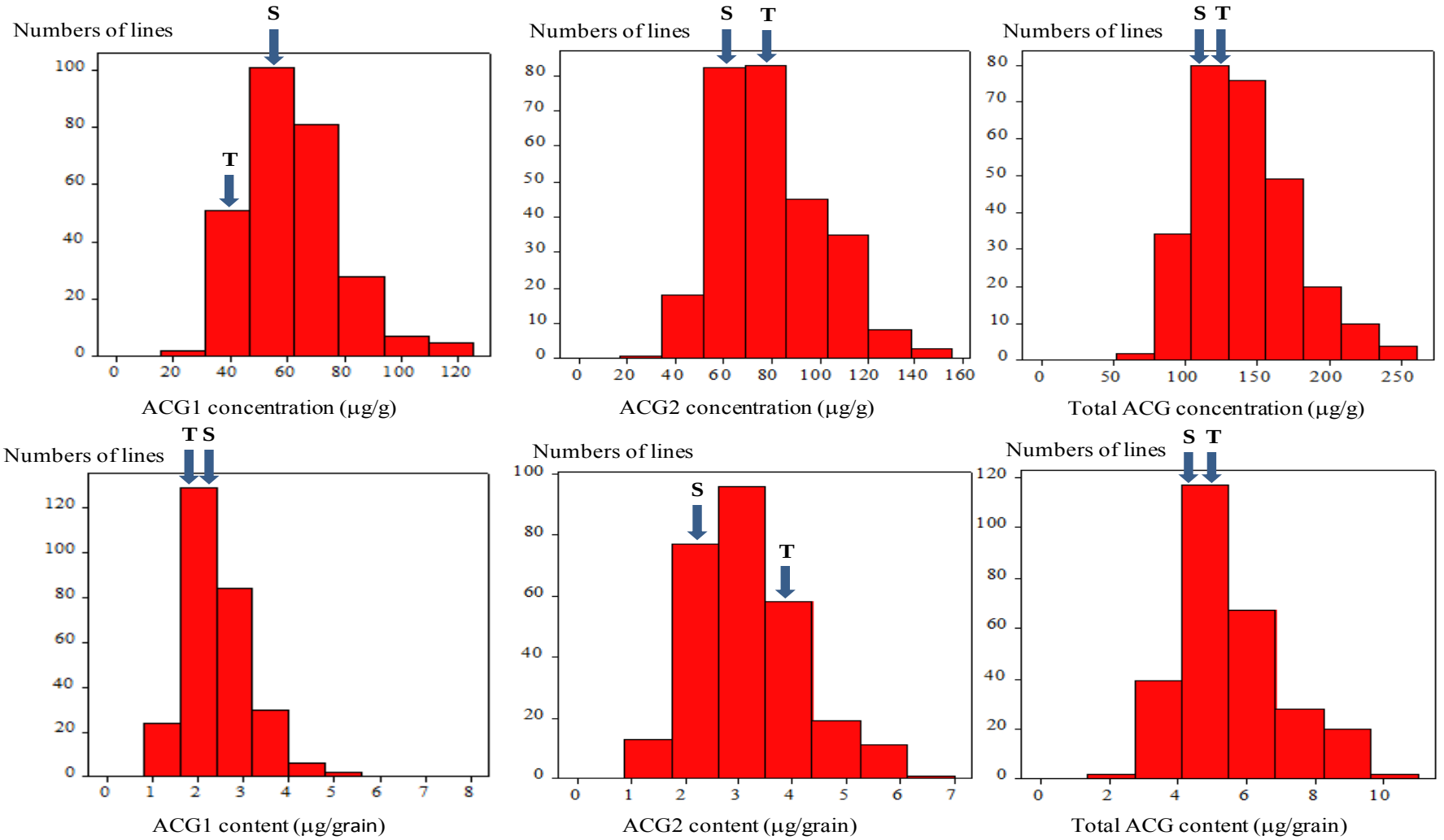


Figure 6.2 Frequency distributions for ACG traits in the Sunco/Tasman doubled haploid population used for fine mapping. The blue arrows indicated the means of the trait for the parents (T: Tasman, S: Sunco).

6.3.2 QTL for ACG content and composition in Sunco/Tasman

The genetic maps of the 21 chromosomes of Sunco/Tasman include a total of 361 markers (Supplemental material 6.1).

The most significant QTL detected was at the wmc76 marker locus at the 18.0 cM of chromosome 7B. At this position, the allele from Tasman had negative effects on ACG1 concentration and content and positive effect on ACG2 concentration and content and thus a strong negative effect on the ACG1/ACG2 ratio. Smaller effects on some of these traits were detected at on chromosomes 4A and 4B. At the cdo795 marker locus at the 5.7-cM on chromosome 4A, the Tasman allele had a negative effect on ACG2 concentration. Near the marker loci gwm495 (at 16.4 cM) and gwm113 (at 14.9 cM) on chromosome 4B, the Tasman allele was associated with larger grain and lower ACG (ACG1, ACG2 and total) concentration) than the Sunco allele. There were also QTL on chromosomes 2B and 4D that affected grain size, with no effect on ACG traits.

QTL located on 7BS for ACG1/ACG2 ratio, ACG1 concentration (µg/g) ACG1 content (µg/grain) and total ACG concentration (µg/g) were associated with a negative additive effect (Table 6.2); in other words, replacing the Sunco allele with the Tasman allele resulted in a reduction in trait magnitude. Conversely, ACG2 concentration (µg/grain) and content per grain were associated with positive additive effects (Table 6.2; supplemental materials 6.2). No significant epistatic interactions were observed. In view of the very high percent of variation in ACG traits explained by the QTL on 7BS, this region clearly warranted further investigation and was selected for fine mapping.

Major QTL for 100 grain weight were located on chromosomes 2B (LOD 5.42), 4B (LOD 9.57), and 4D (LOD 5.68), consistent with an earlier report by Mares and Campbell (2001). The QTL on 2B was located on a chromosome segment translocated from *Triticum timopheevii* (Friebe *et al.* 1996, Kammholz *et al.* 2001) that also contains QTL for flour yield (Lehmensiek *et al.* 2006) stem rust resistance and black point resistance (Lehmensiek *et al.* 2006, Christopher et al. 2007). Within the Sunco/Tasman population, the presence of the Sunco allele at the grain weight QTL-2B was associated with an 8 – 12% reduction in mean grain weight compared with the Tasman allele (Mares unpublished data). Whilst the 2B QTL explained nearly 18% of the variation in grain weight in the current analysis (Table 6.2), it appeared to have little impact, apart from a suggestive effect on total ACG content (μg/grain), on ACG concentration or content (Supplemental material 6.2). By contrast, the 4B QTL that explained nearly 27% of the variation in grain weight had a significant impact on ACG1, ACG2 and total ACG concentration. The 4D QTL by comparison had a significantly weaker effect on grain weight and no effect on the ACG traits.

The marker nearest the peak of the minor QTL on 4B (ACG1 concentration, ACG2 concentration, total ACG concentration, and total ACG content), gwm495, was located close to csME1, a perfect marker derived from the semi-dwarfing gene, *Rht1*. Similarly, the closest marker to the 100 grain weight locus on 4D, csME2, is derived from the other common semi-dwarfing gene, *Rht2*. These genes have been reported to have a pleiotrophic effect on grain size (Pinthus and Gale, 1990; Rebetzke and Richards, 2000). Mares & Campbell (2001) reported an association of a 4B QTL with flour and noodle colour (b*) that could not be related to xanthophyll content and suggested that this was possibly an indirect effect of grain size on milling and flour quality. An SSR

marker, wmc48, located 3cM on the telomeric side of gwm495, was reported to be influence flour yield and kernel hardness (Lehmensiek *et al.* 2006).

Sunco contains the semi-dwarfing gene, *Rht1,* on 4B whilst Tasman has *Rht2* on 4D (Ellis et al. 2002 in Lehmensiek et al. 2006). As a consequence the Sunco/Tasman doubled haploid population consists of lines with tall (*rht1, rht2*) with genotypes BA (Tasman B and Sunco A), semi-dwarf (*Rht1, rh*t2 or *rht1, Rht2*) with genotypes AA and BB respectively and extreme dwarf (*Rht1, Rht2*) phenotype with genotypes AB. Mares and Campbell (2001) reported significant differences in the means of grain size between these phenotype classes. Moreover, as the means of the plant height in 1998 decreased in 1999, the means of the 1000-kernel weight also decreased; from 42.4 to 44.35 for the tall group, from 36.9 to 40.73 for the semi-dwarf and from 30.1 to 33.7 for the dwarf groups.

The 4A QTL for ACG2 concentration explained only 4% of the variation, could not be associated with any previously reported genetic effects on grain size or grain constituents and was not pursued further in this study.

Table 6.2 QTL associated with ACG traits in Sunco/Tasman doubled haploid population. The QTL were backward selected from the genome scan with CIM. All of these QTL are significant at LOD threshold = 3.405, except *.

Chromosomes	Closest markers	Estimated QTL peak position	Traits	LOD	QTL effects		
					%Explain variation	Additive effect	Standard Error
2B	wmc35a	50.6	100 grain weight (g)	9.905	17.762	0.262	0.038
4A	cdo795	5.7	ACG2 concentration (μg/g)	3.793	4.412	-5.733	1.481
4B	gwm495	16.4	ACG1 concentration (μg/g)	3.376	5.119	-5.162	1.431
			ACG2 concentration (μg/g)	3.866	4.524	-5.805	1.482
			Total ACG concentration (μg/g)	3.761	8.93	-10.553	2.741
	gwm113	14.9	100 grain weight (g)	14.196	26.966	0.323	0.037
4D	csME2	0	100 grain weight (g)	5.851	8.928	-0.186	0.037
7B	wmc76	18	ACG1 concentration (μg/g)	15.83	35.408	-13.575	1.458
			ACG1 content (μg/grain)	10.715	26.813	-0.499	0.069
			ACG2 concentration (μg/g)	24.761	49.517	19.204	1.513
			ACG2 content (μg/grain)	20.469	46.411	0.823	0.074
			ACG1/ACG2 ratio	58.893	85.894	-0.241	0.009

6.3.3 Fine mapping of ACG QTL on 7BS of Sunco/Tasman

6.3.3.1 Additional markers

Of the 67 SSR markers that were tested for polymorphism in the Sunco/Tasman doubled haploid population, 27 were polymorphic. However, only 11 were added to the map due to missing genotype data or distortion of the frequency of the marker genotype towards one of the parents (Supplemental material 6.3). Five markers from the long arm of the original map were excluded because the distance between two closest markers from the short arm and the long arm exceeded 90 cM.

Two of the 6 SNP markers, BE352570 and BE445287*,* shown by Dr. Matt Hayden (pers.comm) to be polymorphic between Sunco and Tasman were successfully mapped. Sequencing of the other ESTs: *BE518436*, *BF201699*, *BQ169669*, and *BQ171683* to obtain reference sequences for designing the primers to amplify the SNP were unsuccessful. The *BE352570* contig was successfully sequenced and an allele apecific primer (ASP) was designed (Figure 6.3A). The genome specific primers (GSP) for this EST sequence were used as locus specific primers (LSP) to amplify the EST. The amplified sequences were then re-run with the Allele Specific Primers (ASP). This marker was successfully assayed (Figure 6.3B) in 271 lines of the doubled haploid populations. Two SNP markers were designed from the *BE445287* contig (Figure 6.4A). However, only one of the *BE445287* SNP markers was successfully assayed in 129 lines (Figure 6.4B) and included in the map. The other marker was polymorphic between Sunco and Tasman and in the panel of 20 doubled haploid lines but could not be scored on the majority of the doubled haploid lines (Figure 6.5C).

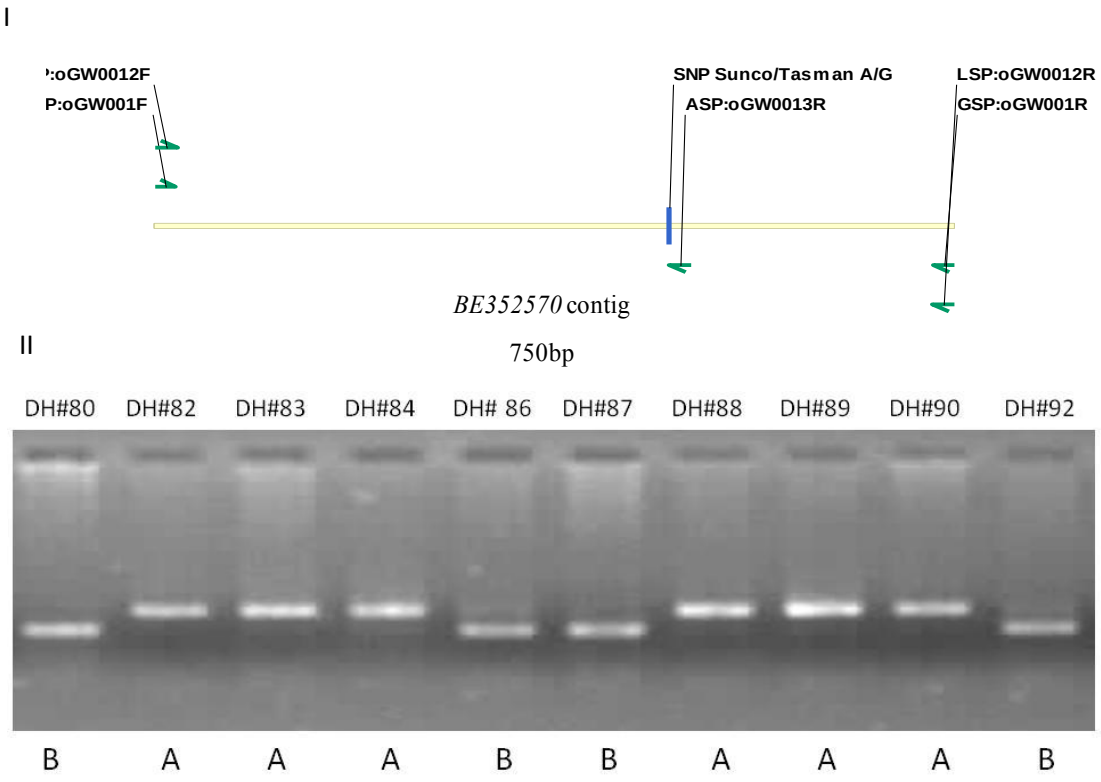


Figure 6.3 Positions of the ASP and GSP primers for the SNP marker derived from the *BE352570* EST contig, I. The design of specific primer (LSP) (genome specific primers that were re-used to amplify the specific EST locus), and allele specific primer (ALS). B. The polymorphisms of the SNP marker within *BE352570* EST (573bp); II. typical banding patterns obtained with some of the doubled haploid lines (#DH80 to #DH92). Bands scored as: A for Sunco alleles and B for Tasman alleles.

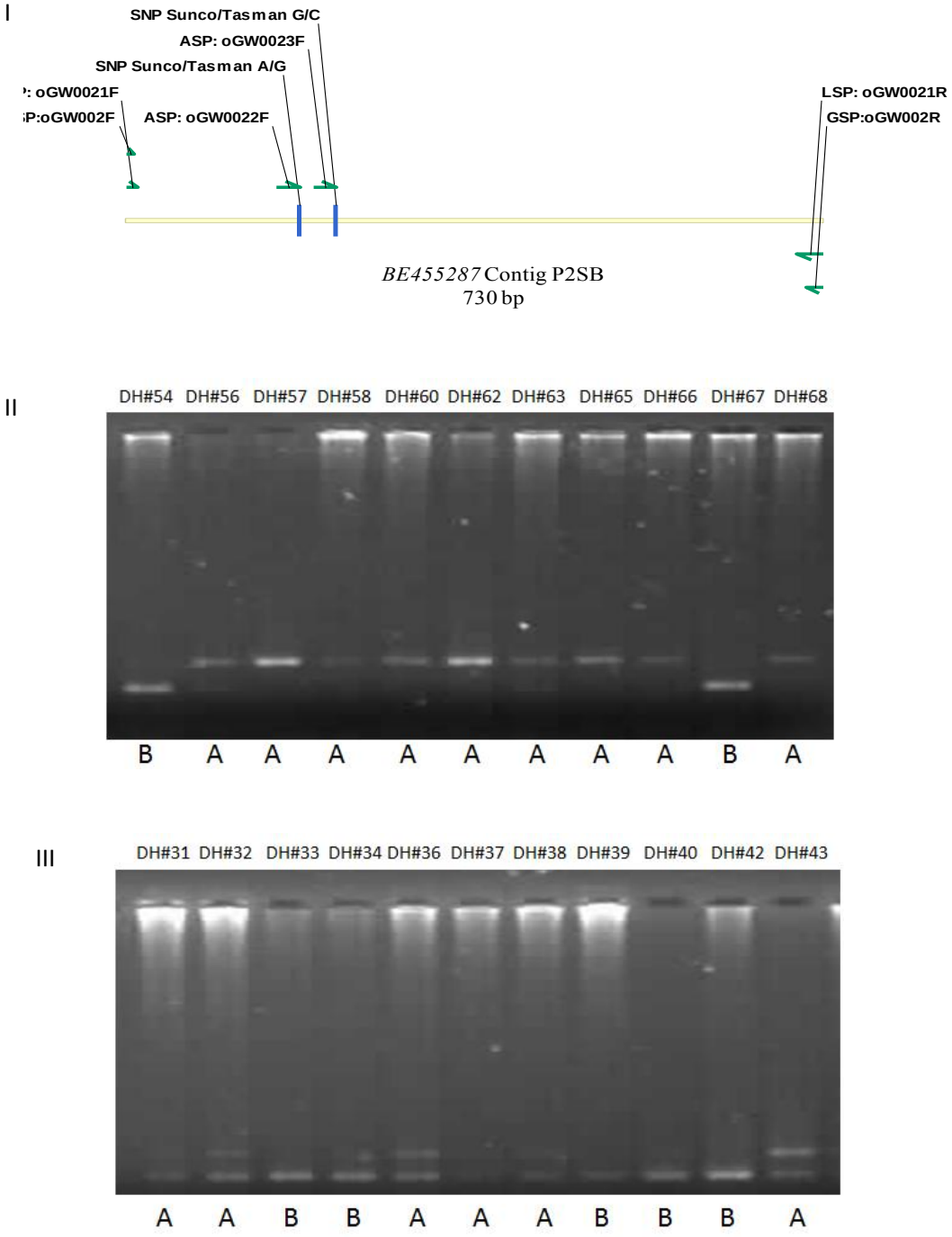


Figure 6.4 SNP markers designed from *BE445287* EST contig. I. The design of specific primer (LSP) (genome specific primers were re-used), and 2 allele specific primers (ALS). II. The polymorphisms of the first *BE445287* SNP markers in some of the doubled haploid lines (#DH54 to 68), III. The polymorphisms of the second *BE445287* SNP markers in some of the doubled haploid lines (#DH31 to 43). Both markers were scored as A and B alleles: A for Sunco alleles and B for Tasman alleles.

6.3.3.2 The order of SSR markers within the QTL on 7BS of Sunco/Tasman compared with other populations and a consensus map

Seven additional markers were mapped into the interval, gwm400 to wmc76, containing the major QTL on chromosome 7BS while 6 other markers were mapped on the centromeric side of this region (Fig. 6.5). These additional markers reduced the confidence interval of the QTL peak from 28.8cM between gwm400 and sun16 to 5.5cM, flanked by wmc426 and sun16.

Prior to fine mapping, the order of the markers on Sunco/Tasman 7BS was similar to that in the maps of Synthetic/Opata (BARC) and Chinese Spring/SQ1 (Figure 6.5). However, this order was not consistent with that in the consensus map, where the wmc76 was located proximal to gwm400. After 13 more markers were mapped into the region, the order of the markers on 7BS of Sunco/Tasman was still similar to Chinese Spring/SQ1, both for the original markers and the additional markers, to the original 7B markers in the Synthetic/Opata map and to the additional markers in the wheat consensus map. However, the order of the additional markers was no longer consistent with that in the Synthetic/Opata (BARC) map.

6.3.3.3 QTL for the ACG traits

Major QTL that affected 5 traits of ACG content, concentration and ratio were confirmed on 7BS between wmc426 and sun16 (an interval of 5.5cM distant) (Table 6.3 and supplemental material 6.4). The peak of the major QTL for ACG2 concentration and ACG1/ACG2 ratio were still very close to wmc76, and explained 15.5% and 48.6% of the variation in these traits respectively. About 0.8 cM distal to this ratio QTL, a major QTL peak for ACG2 content (μg/grain) was detected very close to *wmc426* that

explained 12.9% of the variation. The QTL for both ACG1 concentration and content were detected 4.7cM proximal to wmc76, very close to sun16 explaining 13.8% and 13.6% of variation respectively.

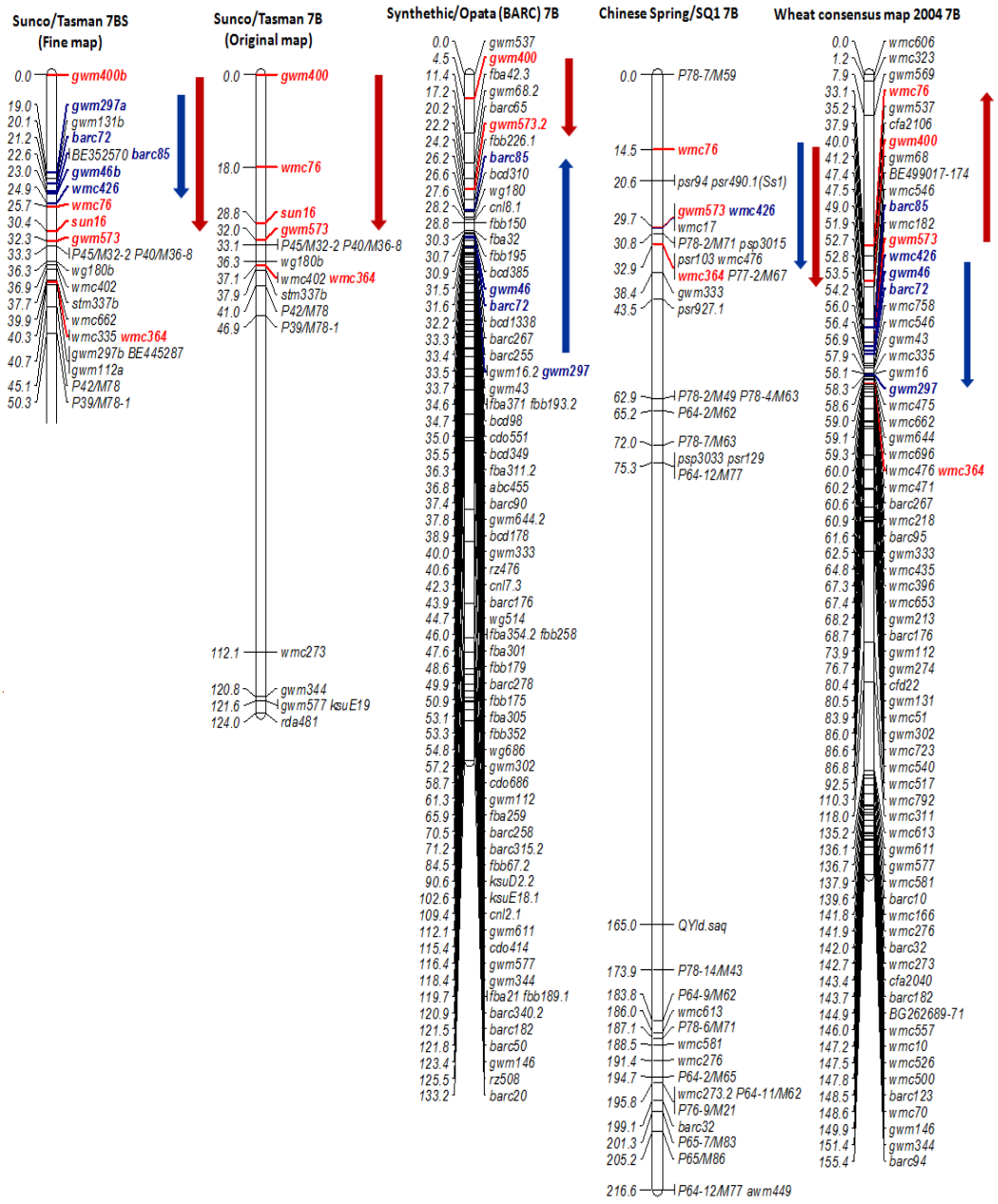


Figure 6.5 Order of markers on 7BS of Sunco/Tasman doubled haploid population before and after fine mapping compared with the order in other genetic maps (Synthetic/Opata (BARC), Chinese Spring/SQ1 and a Consensus map (Somers et al. 2004) sourced from Graingenes databases (access 10 October 2011). Markers and arrows in red are the order of the markers before the fine mapping, while those in blue are the ordered for the additional markers added in this study. Markers in black are other markers in each chromosome that are not in 7BS fine map. Numbering to the left of the chromosomes represents genetic distance (cM).

The ACG1/ACG2 ratio in the Sunco/Tasman doubled haploid population gave a bimodal frequency distribution and was also mapped as a binary trait (A-high ratio/B-low ratio) (Figure 6.6 and Table 6.4). The QTL peak was located between wmc76 and sun16, an interval of 4.7cM, and explained 55.3% of the variation. The difference between explained variation before addition of markers in the QTL region and after was noted. This might be due to the increase in the numbers of phenotypic data values that are in the transitional range between 0.7-0.8 in the data set with 275 doubled haploid lines. There are 27 of them in this data set, while in the 153 doubled haploid lines there was only 1.

Table 6.3 QTL on 7BS of Sunco/Tasman after additional SSR markers were added.

Chromosome	Nearest markers	Estimated QTL peak position (cM)	Traits	LOD	QTL effects		
					%Variation explained	Additive effect	Standard Error
7B	sun16	30.4	ACG1 concentration (μg/g)	9.4	13.8	-6.6	1.0
	wmc0076	25.7	ACG2 concentration (μg/g)	10.9	15.5	8.9	1.3
	sun16	30.4	ACG1 content (μg/grain)	9.3	13.6	-0.3	0.0
	wmc0426	24.9	ACG2 content (μg/grain)	9.1	12.9	0.4	0.1
	wmc0076	25.7	ACG1/ACG2 Ratio	40.9	48.8	-0.2	0.0

Table 6.4 QTL and QTL effect for ACG1/ACG2 ratio analysed as a binary trait A/B (Data output from GENSTAT 14 QTL Analysis, Single Trait Linkage Analysis (Single Environment).

QTL	
Nearest Markers	*wmc76*
Estimated peak position	25.7
Interval	12.8-38.6
LOD	49.1

QTL effect	
% variation explained	55.3
Additive effect	-0.4
Standard error	0.02

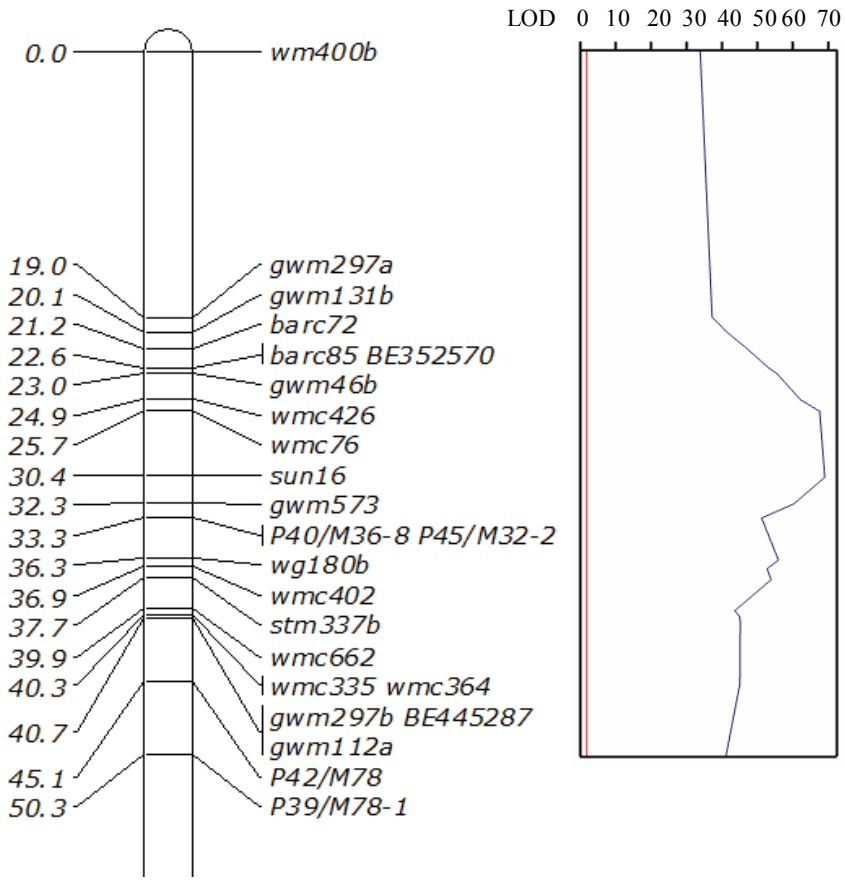


Figure 6.6 QTL on 7BS for ACG1/ACG2 ratio analysed as a binary trait A/B (A: high ratio, B: low ratio). Red line = LOD threshold at Fpr<0.001.

6.3.4 Synteny between the wheat 7BS chromosome region harbouring the QTL associated with variation in ACG traits with the physical maps of rice and Brachypodium distachyon

6.3.4.1 Synteny with rice chromosomes 6 and 8

The 7BS QTL region was aligned to chromosome 6 of rice using EST sequence *BE352570* containing a SNP marker. This marker was located 3.1 cM distal to the QTL peak on 7BS of wheat and the EST was mapped to the short arm of chromosome 6 of rice at the position of (AP005387 PAC/BAC clone) (Fig.6.7). The *BE352570* EST sequence of bread wheat had 88% identity with *LOC_Os06g15990,* annotated as *ALDH2B,* an expressed gene sequence for aldehyde dehydrogenase SALDEHYD-RXN 1:2.1-AST-PWY arginine degradation II, for 82 nucleotides.

This region where AP005387 mapped, also aligned with 2 tightly linked QTL for brown plant hopper resistance (*Bph3* and *Bph4*) on chromosome arm 6S in IR64/Azucena, rice doubled haploid population (Cornell DH QTL 2001, Gramene database). The ACG1 from rice phloem has been reported to be a feeding inhibitor and resistance factor for brown plant hopper, *Nirlaparvata lugens* (Stevenson *et al.* 1996). A bi-functional UDP-glucuronyltransferase and UDP-glucosyltransferase was located 9.727kbp to the centromeric side of *LOC_Os06g15990*. A region that is rich in expressed putative glycosyltransferase genes was also located 6 PAC/BAC clones to the centromeric side of AP005387. There are 11 putative glycosyltransferases (expressed) genes spread across 8 PAC/BAC clones (AP005695 to AP005761) in this region and a basic/helix-loop-helix (bHLH) 86 like protein coded by *LOC_Os06g16400* at AP003044, 3 BAC

clones from the syntenic locus. The complete list of expressed proteins and putative proteins in this region is contained in supplemental material 6.5.

The chromosome region immediately to the centromeric side of the 7BS QTL was aligned to rice chromosome arm 8L using *BE445287* SNP marker (Fig. 6.8). The *BE445287* EST sequence was located 3.6cM to the centromeric side of the 7BS QTL region and aligned with *LOC_Os08g40530* at AP004761 PAC/BAC clone of rice 8L (93.8% identity).

LOC_Os08g40530 on rice 8L expressed a putative calcium-transporting ATPase 9, plasma membrane-type protein. Other putative genes of interest in rice PAC/BAC clones close to the SNP marker, included dihydroflavonol 4-reductase (DFR) (*LOC_Os08g40440,* AP004761), 5 cytochrome-P450 monooxygenase genes spread across 3 PAC/BAC clones (AP005816 to AP005254), and a sequence containing a basic/helix-loop-helix (bHLH) 91 DNA binding domain. The putative protein sequences expressed by the genes located in these clones are listed in supplemental material 6.6.The interaction between myb and bHLH transcription factors was reported to result in the activation of branches of the flavonoid pathway that compete for precursor required for the ACG biosynthesis and which which lead to anthocyanin, proanthocyanin and flavonol glycoside biosynthesis (Bogs et al. 2007, Goff et al. 1992, Nakatsuka et al. 2008). These transcription factors were reported to up-regulate DFR genes involved in producing red seed coat colour (Himi et al., 2005).

When recombinants were arranged in order from the distal end of the chromosome towards the centromere (as described for boron tolerance in Schnurbusch et al. 2006), a change from Sunco allele to Tasman allele between wmc76 and sun16 was associated

with a change in ratio from Sunco type to Tasman type. These markers appear to be much closer to the SNP linking 7BS with rice chromosome 6S than the SNP linking 7BS with rice chromosome 8L. As a consequence, the synteny with rice chromosome 6S would seem to be more relevant to control of variation in ratio and the glycosyltransferase(s) more likely as the candidate gene for ACG1/ACG2 ratio than the transcription factors.

The region containing the minor QTL on 4B for ACG content in Sunco/Tasman was syntenic with rice chromosome 3 (supplemental material 6.7). The synteny analysis was performed *in silico* by aligning the Sunco/Tasman QTL region with the wheat physical map, SSR (Grain genes 2003), then aligning this second map with the wheat physical map, EST (Grain genes 2003). ESTs that were located in the predicted deletion bin containing the QTL were used to align the deletion bin with rice chromosome 3. Although the region around the 4B QTL of Sunco/Tasman is quite dense with markers, with 5 markers within 4.5cM; the syntenic region in rice chromosome still covered a large section (15-27Mbp). In addition, given that this QTL appears likely to be co-located with the semi-dwarfing gene, *Rht1*, it is probably worthwhile to examine this region in other bread wheat populations, where parents show greater variation for total ACG concentration and grain content.

The wheat-rice synteny seemed not to be as direct and continuous as previously explained by (Sorrells et al. 2003). Disruptions by homology of small numbers of genes with other chromosomes, genomic sequences from other chromosomes were observed, e.g. wheat 7B - rice 6 is interrupted by a section of rice chromosome 5, and there are genes/wheat sequences on wheat 3B that are syntenic with rice chromosome 6. Four

wheat EST sequences at 3B are similar to 8 sequences on rice 6S while 3 others are similar to 7 sequences on 6L (Supplemental material 6.8). Wheat EST *BF485490* (729,556,473bp at 3BL) is syntenic with a block of sequences between AP004725 (7,634,956kb) and AP004651 (7,636,291kb), from *LOC_Os06g13640* to *LOC_Os06g13910*. Two glycosyltransferases were located at *LOC_Os06g13710* and *LOC_Os06g13760*. The complete list of these sequences is presented in Supplemental material 6.8. The occurrence of these disruptions have also been reported by (Francki et al. 2004).

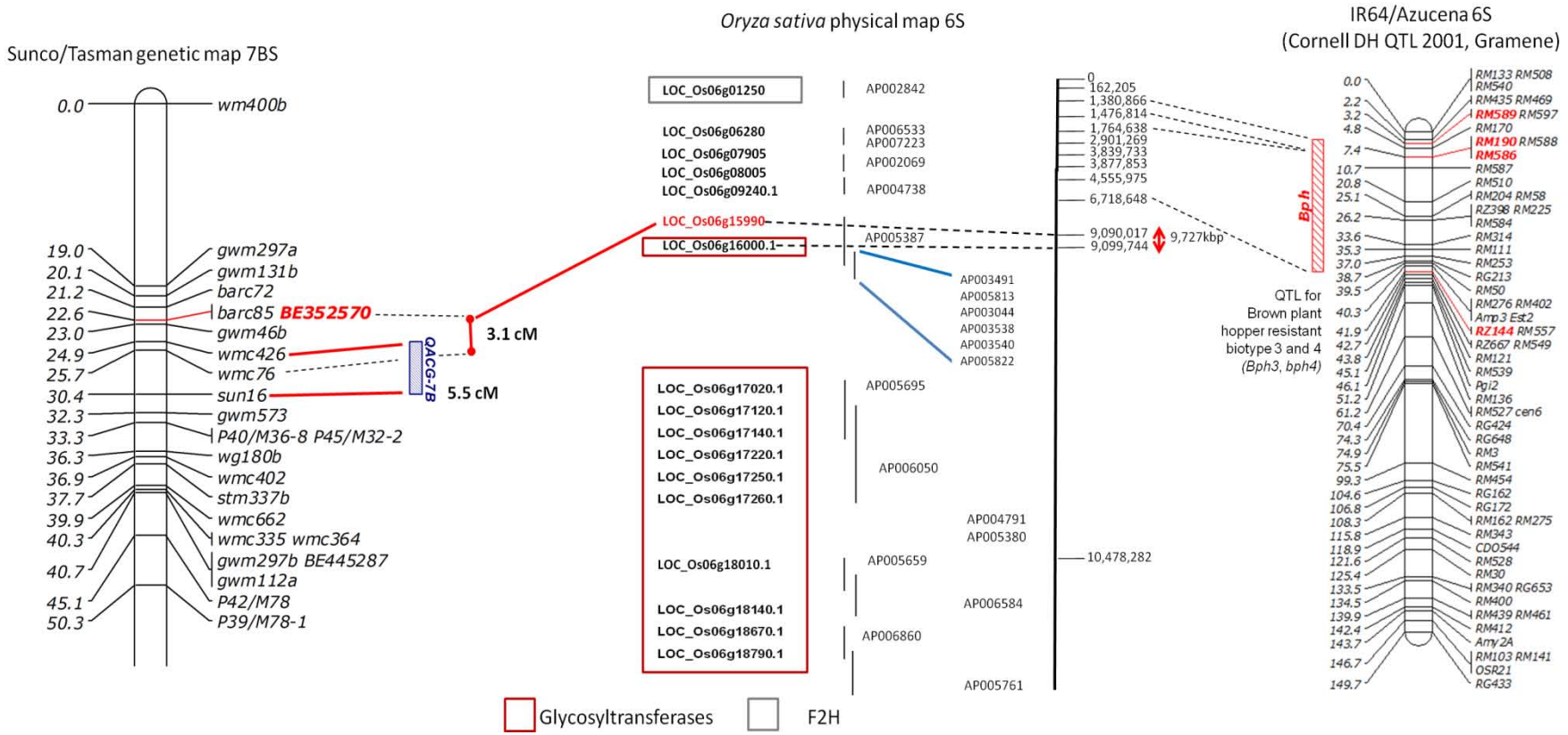


Figure 6.7 Comparative map of 7BS Sunco/Tasman, rice physical map 6S and rice RI64/Azucena 6S. The markers and loci in red are markers that link 7BS and rice 6S of physical map and markers that flank the QTL region in rice. Locus in grey box: F2H, loci in red boxes: glycosyltransferases. Blue bar *QACG-7B*: QTL for ACG trait at 7B, red bar *Bph*: QTL for brown plant hopper resistance. Numbers on the left of Sunco/Tasman 7BS and rice IR64/Azucena maps show the genetic distance between markers (cM), while those on the left of rice physical map 6S show the distancein kilo base pairs (kb). On the Sunco/Tasman map, 3.1cM is the distance between SNP marker *BE352570* and wmc76, nearest marker to ratio QTL, while 5.5 cM is the size of the chromosome intervalL between wmc426 and sun16. Information on the rice physical map 6S and rice RI64/Azucena 6S genetic map was obtained from the Gramene database.

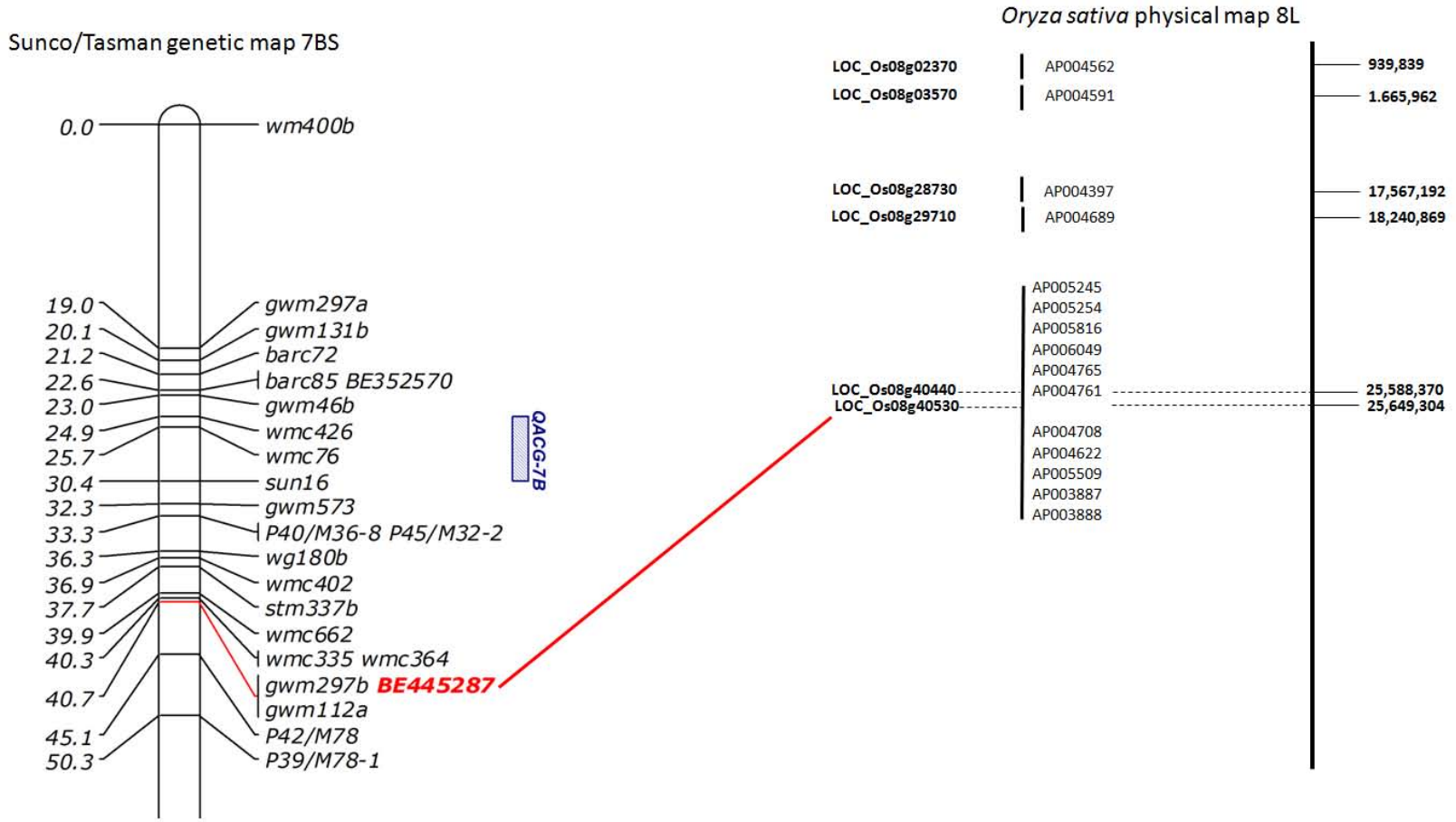


Figure 6.8 Comparison of Sunco/Tasman 7BS with the physical map of rice 8L (Gramene database). The 7BS marker in red is the wheat EST SNP marker used to link the two maps. The blue bar marked *QACG-7B* is the QTL region on 7BS for ACG traits (Table 6.3).

6.3.4.2 Synteny with *Brachypodium distachyon* chromosomes 1 and 3

The 7BS chromosome arm of wheat is syntenic with chromosomes 1 and 3 of *Brachypodium distachyon*. The SNP marker from EST BE352570 linked 7BS of Sunco/Tasman with chromosome 1 of *Brachypodium distachyon* (Figure 6.9) at *BRADI1G43770* locus (41.5Mbp), while that from EST *BE455287* linked 7BS with chromosome 3 (Figure 6.10) at *BRADI3G40640* (42.7Mb). A block of loci in rice chromosome 6S (2.6Mbp-13Mbp) was syntenic with *Brachypodium distachyon* chromosome 1 (Os6_Bd1_R.1: 39.8Mbp-48.1Mbp) (supplemental material 6.9 and 6.10), while, a block of loci on rice chromosome 8L (19.4Mbp-28.1Mbp) was syntenic with *Brachypodium distachyon* chromosome 3 (Os08_Bd3_F.1: 37.8Mbp-44.4Mbp) (supplemental material 6.11).

The relationship between wheat 7BS genetic map and *Brachypodium distachyon* physical map chromosome 3 was established through the synteny between *Aegilops tauschii* reference map (Dvorak et al. 1995) and *Brachypodium distachyon* comparative map. Berkman et al. (2012) established a syntenic build between wheat homoeologous group 7 and *Brachypodium distachyon* chromosome 1. Approximately 13% of the genes on 7BS have been translocated to 4AL, and this translocation region that was marked by *BRADIg49510* to the telomere showed a similar gene content to 7BS and 7DS (Berkman *et al.* 2012). 7DS and 7BS sequences distal to this translocation region were highly conserved. No information is available yet for the syntenic build between group 7 and chromosome 3. The SNP marker *BE455287* at 7BS Sunco/Tasman was not available in *Brachypodium distachyon* chromosome 3, and does not have corresponding EST cluster in the comparative map. However, such information was available for 7DS.

The glycosyltranferase sequences homologous to those on rice chromosome 6S are located on chromosome 1 of *Brachypodium distachyon*, but were ordered in the opposite direction to rice. Four rice glycosyltranferases, *LOC_Os06g17250*, *LOC_Os06g17220*, *LOC_Os06g17140* and *LOC_Os06g17120,* were aligned to *BRADI1G43600* (41.4Mbp), while 3 others, *LOC_Os06g18670, LOC_Os06g18790,* and *LOC_Os06g17020,* were aligned to *BRADI1G44630* (42.7Mbp). No loci harboring sequences homologous to LOC_Os06g17260 or LOC_Os06g18140.0 were found. Only 4 out of 11 glycosyltransferases in the region of rice syntenic to 7BS were identified in *Brachypodium distachyon* chromosome 1.

Epimerases, galactosyltranferases and DFR gene sequences identified in chromosome 8L of rice were also in *Brachypodium distachyon* chromosome 3. The order of loci are very similar, but rice chromosome has more loci that has been annotated and syntenic with wheat than the *Brachypodium distachyon* chromosome.

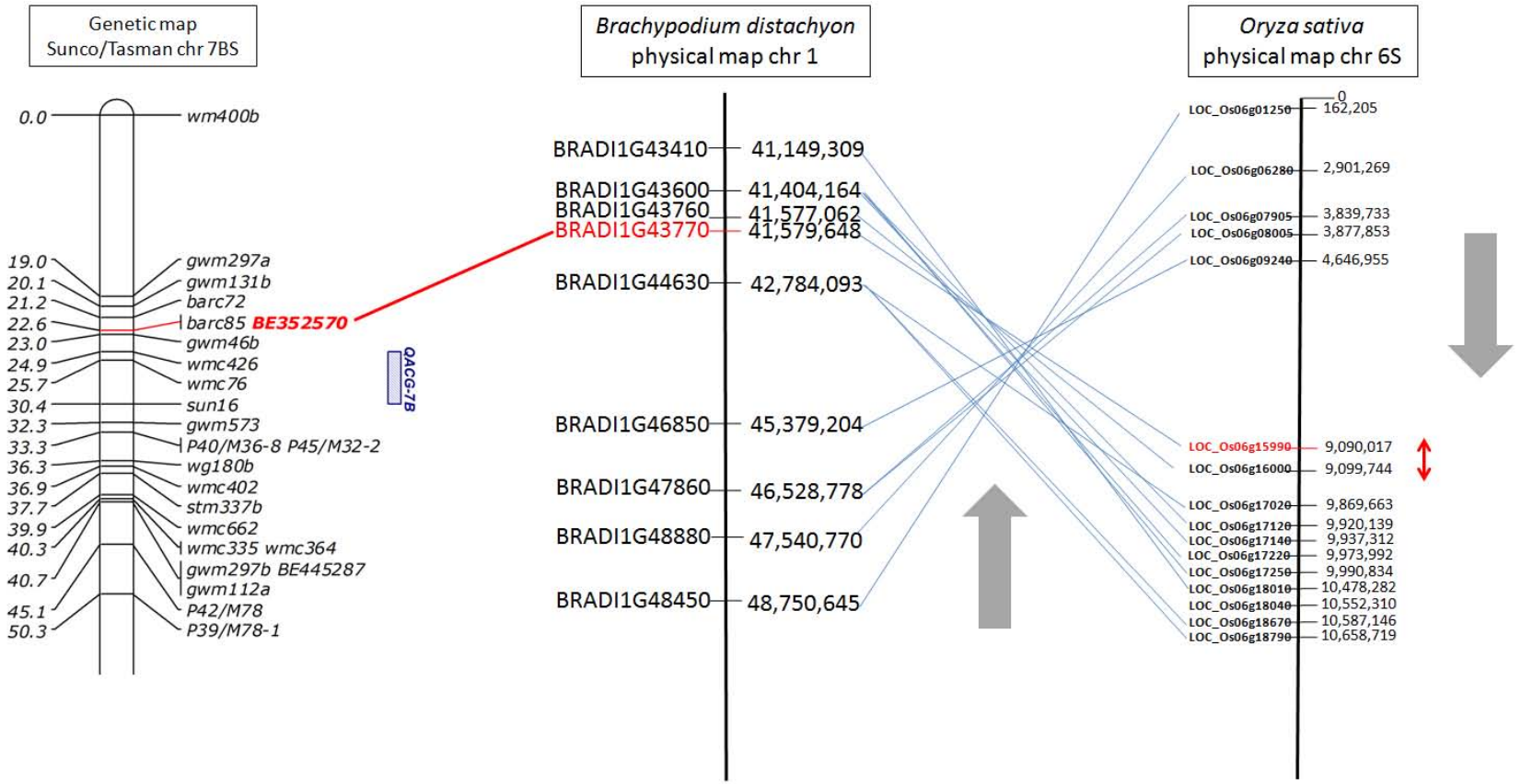


Figure 6.9 Comparison of the Sunco/Tasman 7BS genetic map, with the *Brachypodium distachyon* physical map of chromosome 1 and the rice physical map of 6S (Gramene database). Loci in red indicate the linkage between maps. The arrows show the order of the loci. The physical maps are based on information from Gramene database. Blue bar *QACG-7B*: QTL for ACG traits at 7BS. Numbers on the left of Sunco/Tasman 7BS show the genetic distance between markers (cM), while those on the right of *Brachypodium distcahyon* and rice physical maps show distance in kilo base pairs (kb).

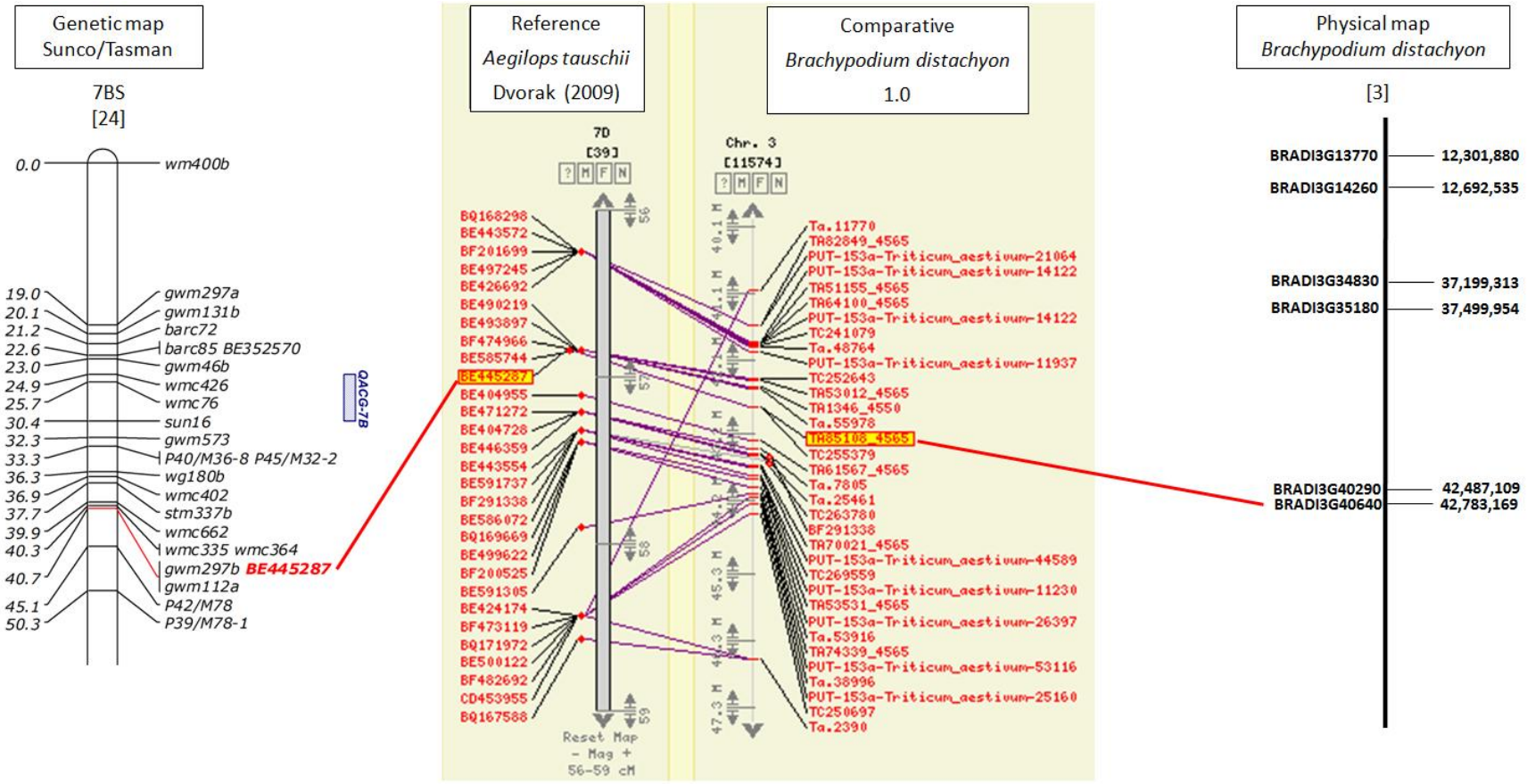


Figure 6.10 Comparison of the maps for Sunco/Tasman genetic map 7BS, *Aegilops tauschii* 7D (Reference map of Dvorak (2009), *Brachypodium distachyon* chromosome 3 (Comparative map 0.1, and physical map (Gramene Release 31) were obtained from Gramene database). Blue bar *QACG-7B*: QTL for ACG trait at 7BS. Marker in red (*BE445287*) at 7BS Sunco/Tasman link this population map to *A. tauschii* reference map, which structural relationship with the *Brachypodium distachyon* comparative map has been established. Red lines showed the links, while markers in yellow boxes are the markers that link the *A. tauchii* reference map and the *Brachypodium distachyon* map. The purple lines show the relationship between the wheat EST in the wheat map and annotated EST cluster in the *Brachypodium* map.

6.3.5 Candidate genes related to variation in ACG biosynthesis in wheat

Based on the biosynthetic pathway, there are several types of genes that might be candidates for regulating the ACG biosynthesis. These genes include those early in the common pathway to ACG1 and ACG2. Firstly, the transcription factors that have been reported for flavonoid biosynthesis. They generally activate large sections of the pathway not individual steps. Secondly, the genes that function later in the pathway. They are more likely to determine the ratio and content of ACG1 and ACG2. This particular part of the pathway involves the transfer of glucose and galactose molecules to the open hemiketal ring structure. In addition, both ACGs contain arabinose, and hence epimerases and sugar transporters may be important in controlling the relative concentrations of UDP sugars.

6.3.5.1 The glycosyltransferases

The phylogenetic tree of these putative glycosyltransferases is presented in Figure 6.11. These 11 sequences were grouped into 2 main clades. The first clade (in the blue box) was closer to the previously reported plant *O*-glycosyltransferases: UDP-glucosyl and glucuronosyl transferase of maize bronze alleles *Bz-W22* (Furtek et al. 1988, Ralston et al. 1988), UDP-glucose:flavonoid 3-O-glycosyltransferase with ability to transfer UDP galactose in *Vitis vitifera VvGT1* (Offen *et al.* 2006), UDP-glucuronic acid:flavonol-3-O-glucuronosyltransferase (GAT) of *Vitis Vitifera VvGT5* (Ono *et al.* 2010), and UDP-glucose/UDP-galactose:flavonol-3-O-glucosyltransferase/galactosyltransferase of *Vitis Vitifera VvGT6* (Ono *et al.* 2010). The *C*-glycosyltransferase of rice (*OsCGT*) that was previously reported by Brazier-Hicks et al. (2009) together with its orthologue in *Brachypodium distachyon* (*BRADI1G43410*) and *Sorghum bicolor* were also in this group.

The second group of 5 putative UDP-glucose glycosyltransferases (in the red box) has similar amino acid sequences to the *O*-glucosyltranferases reported at V25L and W168S of the N-terminal domain, that are polymorphic with the first group containing *C*-glycosyltransferase and its 2 orthologues (Supplemental material 6.10). The amino acid signature of the plant secondary product glycosyltransferase motif (PSPG) of these 11 sequences is conserved, except for the Gln-44 (Q) (Figure 6.12). Polymorphism was observed in the first clade that contained the rice *C*-glycosyltransferase and its ortholog. Two sequences, *LOC_Os06g18670* and *LOC_Os06g18790* have histidine instead of glycine. The last two amino acids of the PSPG motif Glu43 (D) and Gln44 (Q) interact with glucose moiety of the sugar donor, through formation of hydrogen bonds with its 2-, 3-, and 4-OH (Osmani et al. 2009, Wang 2009). The mutation of H374Q switched sugar donor preference from UDP-galactose to UDP-glucose (Kubo et al. 2004). But, this does not appear to work the other way around, since the change abolished activity with both of the sugars (Offen et al. 2006).

The N-terminal domain is less conserved than the C-terminal domain and is involved in determining sugar donor specificity (Osmani et al. 2009). The polymorphisms between the *C*-glycosyltransferase-like sequence group and the putative O-glycosyltransferase were located at the beginning of the sequence Nβ1 (before the loop N1) and at loopN4 (Figure 6.13). These loops have been reported to be crucial for the sugar acceptor pocket formation (Brazier-Hicks et al. 2007, Li et al. 2007, Offen et al. 2006, Osmani et al. 2009, Shao et al. 2005, Wang 2009).

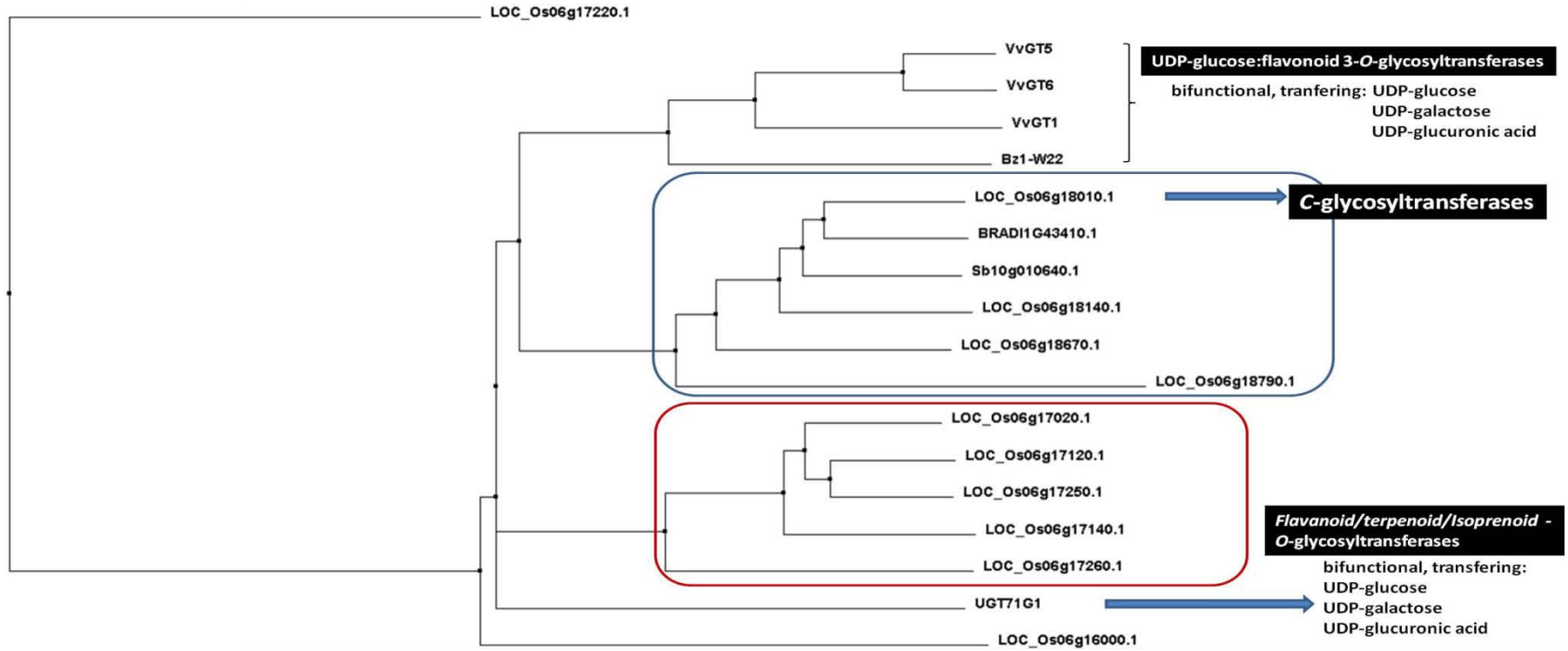


Figure 6.11 Phylogenetic tree of the putative UDP-glycosyltransferases in the region of rice chromosome 6 that is syntenic with the ratio QTL on Sunco/Tasman compared with other UDP-glycosyltransferases: Bz1-W22 (Maize UDPG-flavonoid 3-*O*-glucosyltransferase), BRADI1G43410.1 and Sb10g010640.1 (*Brachypodium distacyon* and *Sorghum bicolour* glycosyltransferases that were the orthologs of rice *C*-glycosyltransferase (OSCGT, LOC_Os06g18010.1), UGT71G1(*Medicago truncatula* flavonoid/triterpene *O*-glucosyltransferase, VvGT5 (*Vitis vitifera* UDP-glucuronic acid:flavonol-3-*O*-glucuronyltransferase), VvGT6 (*Vitis vitifera* bifunctional UDP-glucose/UDP-galactose: flavonol-3-*O*-glucosyl/galactosyltransferase) and VvGT1 (*Vitis vitifera* UDP-glucose:flavonoid 3-*O*-glycosyltransferase). The cereal protein sequences were obtained from Gamene website, while the other protein sequences were obtained from NCBI databases. Blue box: first clade, red box: second clade.

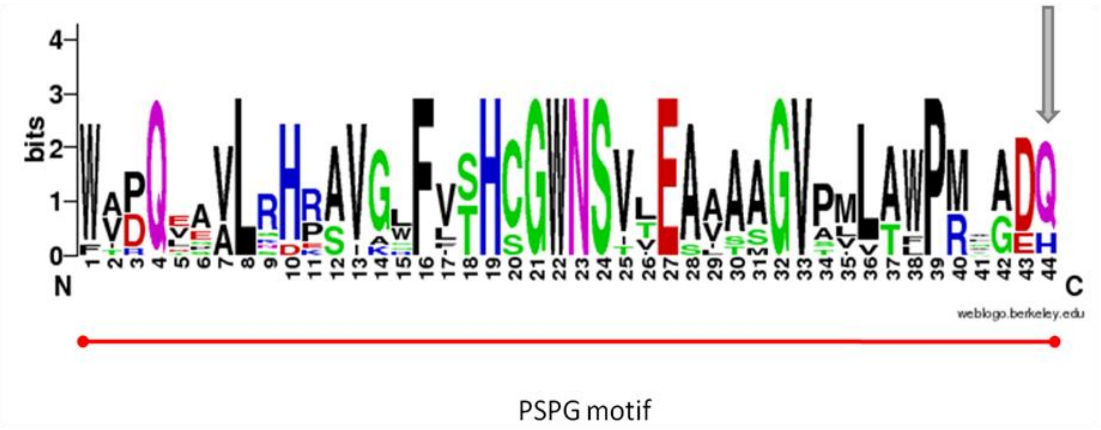


Figure 6.12 Conserved region of the Plant Secondary Product Glycosyltransferase (PSPG) motif of the putative UDP-glycosyltransferases from the region of rice chromosome 6 that is syntenic with the ratio QTL on 7BS of Sunco/Tasman. N and C at the x axis of the weblogo showed the direction from N-terminal to C-terminal of the protein sequence; the height of the letter stacks showed the conservation of the amino acid, and the height of the letters within the stack indicates the relative frequency of each amino acid at that position. The arrow shows the amino acid polymorphism for UDP-glucose recognition and UDP-galactose recognition (D). The D463H mutation converted the bifunctional UDP-glycosyltransferase to a monofunctional galactosyltransferase (Kubo et al. 2004, Offen et al. 2006, Ono et al. 2010, Osmani et al. 2009).

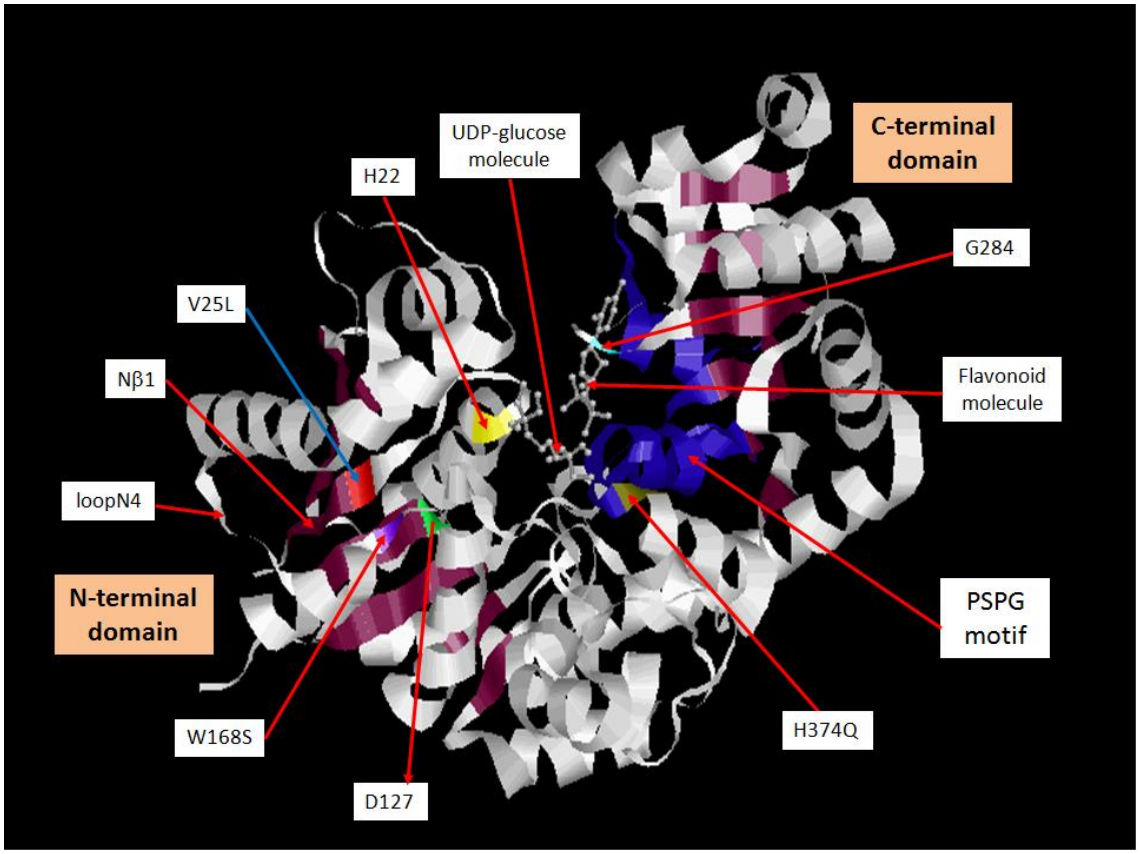


Figure 6.13 Model of VvGT1 and position of the amino acid highlighted in supplemental material 6.12. The red-maroon ribbons show the β-helix structure position, while the white ribbon shows the α-helix and loop structure position. The dark blue ribbon shows the position of the PSPG motif. Small segments of the ribbon in red, purple and gold colour show the positions of the amino acid polymorphism, while those in light blue, green and yellow show the position of the conserved amino acids. The ball and stick structure in the middle of the stereo molecule are the flavonoid and UDP-glucose substrate molecules. The VvGT1 backbone was re-drawn from the PDB file for the UDP-glucose: flavonoid 3-O-glycosyltransferase of VvGT1 (2CIX) (Offen et.al. 2001) and this file was obtained from RSCB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The 3D secondary structure was re-drawn from Osmani et al. (2009), while the conservation and polymorphism of amino acids were drawn from Shao et al. (2005). Information for the protein domain, family and functional sites of the plant O-glycosyltransferases were downloaded from Prosite database of ExPASy Swiss Institute of Bioinformatics (SIB) Bioinformatics Resource Portal (http://prosite.expasy.org/), while the correlation between the multiple alignments and the structural information of the PDB file were obtained from the S2C facility (http://www.fccc.edu/research/labs/dunbrack/s2c). Further details are explained in the text.

6.3.5.2 Other candidate genes for the major and minor QTL associated with ACG content and composition.

A putative F2H gene, *LOC_Os06g01250,* is located at the distal end of chromosome 6S (start at 162,205bp). This gene is a candidate for the first part of the proposed biosynthetic pathway for ACG (Figure 2.5) where naringenin flavanone is converted to 2-hydroxy flavanone. F2H activity may be involved in the control of the total concentration and total content of ACG.

Other candidate genes possibly related to the availablity of the UDP-sugars required by the glycosyltransferases are genes coding for epimerases or UDP-sugar transporters. Genes coding for two putative expressed NAD dependent epimerase/dehydratase family proteins (Q8H0B2) with putative UDP-arabinose/glucose 4-epimerase 3 activity (that catalyses the conversion of UDP-galactose to UDP-glucose), *LOC_Os08g03570* and *LOC_Os08g28730,* were located in PAC/BAC AP004591 and AP004397 respectively (Figure 6.8). The *LOC_Os08g03570* locus is a homolog of *BE498418* wheat EST at C-7BL2-0.33 in the C-7AL1-0.39 deletion bin of Chinese Spring. The homologies are observed at exons 5, 6, and 7 with 87%, 91% and 93% sequence identity for 89, 140, and 148 nucleotides. The wheat and rice exons are generally conserved, while the introns have less similarity, as previously reported in cereals (Bossolini *et al.* 2007, Li *et al.* 1999)

A wheat EST, annotated as a glucose-6-phosphate transporter (*BE443396*, Q7XY15), located in the deletion bin C-7BS1-0.27 that contains the major ACG QTL at 7BS of Sunco/Tasman, is a homolog of *LOC_Os08g08840* (AP004656), located on chromosome 8 of rice (start at 5,138,640bp) (Figure 6.8). There are two exons in this

genomic sequence. One exon had 89.63% identity (for 241 nucleotides of the 3rd exon) and the other was at 91% identity (for 59 nucleotides of the 2nd exon) with the wheat EST.

A wheat EST annotated as a galactose transporter, *BE498418*, located within the C-7BL2-0.33 deletion bin of Chinese Spring is a homolog with 89 % identity (for 113 nucleotides) of exon 7 of *LOC_Os05g02490* of rice chromosome 5. In addition, two putative expressed galactosyltransferase family proteins were located on chromosome 8 of rice, *LOC_Os08g02370* (AP004562) and *LOC_Os08g29710* (AP004689). There are no other glycosyltransferases located in chromosome 8 of rice.

6.4 Conclusion

The 7BS QTL was saturated with SSR markers to reduce the size of the interval and was shown to be syntenic with a region on chromosome 6 of rice. Eleven putative glycosyltransferases, an *F2H*, 2 *bhLH* transcription factors, 2 epimerases, and sugar transporters located in this region on rice chromosome 6 were identified as potential candidate genes controlling the variation in ACG biosynthesis in bread wheat grain.

There are 2 clades of glycosyltransferases on rice 6S region that differ in a single amino acid at the sugar recognition site. The *O*‐ and *C*‐glycosytranferases differ at V25L and W168S, very close to, or involved in, the sugar donor pocket. The *C*-glycosyltransferase is a possible candidate for the gene controlling the ratio trait based on the combined information from the nullisomic-tetrasomic Chinese Spring analysis, 7BQTL, synteny analysis and C-glycosyltransferases sequence and 3-D structure analysis. This hypothesis could be validated by cloning the homologous wheat 7B *C*-

glycosyltransferase, identifying SNP or other markers between Sunco and Tasman followed by mapping the polymorphisms in the Sunco/Tasman. Moreover, it should also be possible to follow the expression of the gene in developing grain of Sunco and Tasman and to determine its temporal relationship to the synthesis of ACG.

Assuming that the candidate gene within the 7BS QTL is a glycosyltranferase then there are at least 2 models that could explain the observed variation in ACG composition. The first model is based on the Chinese Spring nullisomic-tetrasomic data presented in Chapter IV where the absence of chromosome 7B was associated with an increase in ACG1/ACG2 ratio similar to that found in Sunco and related cultivars. If the glycosyltransferase located on 7BS has a higher specificity for UDP-galactose than UDP-glucose; then in its absence, the flow of intermediates might be directed towards synthesis of the glucose containing ACG, ACG1, assuming that glycosyltransferase(s) located elsewhere in the genome have a higher specificity for UDP-glucose. In this first model, Tasman would have the wild-type allele coding for a glycosyltransferase with higher specificity for UDP-galactose whilst Sunco would have either a null allele or a mutation that significantly reduced the activity of the encoded glycosyltransferase. The second model would involve Tasman having a wild-type allele coding for a UDP-galactose specific glycosyltransferase whilst a mutation in the Sunco allele would be associated with a switch in specificity from UDP-galactose to UDP-glucose.

By analogy, the very low ACG1/ACG2 ratio observed in the Chinese Spring nulli-7D lines (Chapter IV) would suggest that there was a gene coding a UDP-glucose specific glycosyltransferase located on 7DS.

Major QTL were detected on 7BS for a number of ACG traits whilst minor QTL were detected at 4B and 4A. The QTL at 4B appeared to coincide with a QTL for grain size for which the most likely candidate gene is *Rht1*.Whilst it is possible that there is a specific gene within this QTL that directly affects ACG synthesis, it appears more likely that the observed effect on ACG concentration is a pleiotrophic effect of the semi-dwarfing gene.

The results have a number of implications for variety improvement for YAN and further research. The QTL for total ACG content could be validated in other populations and the closely linked markers used in marker-assisted selection. Unfortunately, as shown in Chapter V, simply increasing total grain ACG does not result in higher flour ACG. A longer term solution would perhaps be to transform a bread wheat cultivar, preferably one which already has excellent quality for YAN, with a combination of F2H and glycosyltransferase genes attached to an endosperm specific promoter in an attempt to get ACG synthesis in the starchy endosperm. Further research effort could be focused on cloning the candidate glycosyltransferase gene, validating its role in ACG synthesis and confirming the UDP-sugar specificity. An additional area for research would be the mechanism involved in the apparent link between grain size and ACG concentration and content; and the effect of the plant external factors such as environmental conditions to this link.

Chapter VII: Identification of SNPs and haplotypes in candidate genes for Apigenin di-*C*-glycoside (ACG) biosynthesis in bread wheat (*Triticum aestivum* L.) grain: *C-glycosyltransferase* (*CGT*) and *flavanone 2-hydroxylase* (*F2H*)

7.1 Introduction

In the previous chapter, several possible candidate genes for the control of ACG biosynthesis in bread wheat grain were identified, *C-glycosyltransferase* (*CGT*) and flavanone *2-hydroxylase* (*F2H*). Various PCR based methods have been used to isolate, purify and clone either *F2H* or *CGT* genes. Hicks *et al.* (2010) successfully cloned *CGT* genes from rice genomic DNA (gDNA). In other studies by Ono *et al*. (2010) and Noguchi *et al*. (2009) with the dicotyledonous plants (*Vitis vitifera* and *Lamiales* respectively), *flavonoid glycosyltransferase* genes from the dicotyledones were cloned from the complimentary mRNA via cDNA. Du *et al.* in 2009 and 2010 isolated *F2H* from cDNA of rice and sorghum. Neither *F2H* nor *CGT* have been cloned from bread wheat.

However, other flavonoid pathway genes have been cloned in hexaploid bread wheat and these include *F3H* (Khleskina et al. 2008), *DFR* (Himi et al. 2004) and *R* (*myb* transcription factor) (Himi et al. 2005). In all of these studies, multiple copies of the genes were obtained by PCR amplification. Four copies of *F3H* were obtained from cDNA of nullisomic-tetrasomic (NT) lines Chinese Spring: 1 copy from 7A chromosome, 2 copies from 7B and 1 copy from 7D (Khleskina et al. 2008).

Meanwhile, Himi *et al* in 2004 and 2005 recovered three homeoforms of *DFR* and *R* (*myb* transcription factor) genes from gDNA of NT lines Chinese Spring group 3, one from each of 3A, 3B, and 3D NT lines.

Having identified potential candidate genes controlling variation in ACG composition in wheat, the next step is validation. One strategy for achieving this is to isolate and sequence the genes, then to look for sequence differences between the parents of the mapping population. Any sequence differences can be used to develop markers that can be mapped on the population and marker-trait linkage tested. This chapter describes attempts to amplify *CGT* and *F2H* sequences from genomic DNA and cDNA of bread wheat. Degerate primers were designed based on protein sequence alignment rather than specific DNA primers for the EST, especially with the CGT, because of the disimilarity among their DNA sequences. It is expected that similar protein with identity greater than 35% will have similar functions especially when the functional domains are homolgous. Unfortunately due to time constraints and difficulties in working with a very large gene family in hexaploid wheat, this was not successfully completed and only a progress report is presented here.

7.2 Materials and Methods

7.2.1 Plant Materials

7.2.1.1 gDNA

The wheat genomic DNA template used in this experiment were extracted from the nullisomic-tetrasomic lines of Chinese Spring group 7, Sunco and Tasman parents, Sunco and Tasman panel lines; as explained in details in Chapter III section 3.2.3.3. The

rice genomic DNA used as positive control in this experiment was kindly provided by Rice Salt Group (ACPFG).

7.2.1.2 mRNA

To obtain wheat RNA, the nullisomic-tetrasomic lines of Chinese Spring group 7, Sunco, Tasman, Gamut and Kennedy were grown in the glasshouse during August-December 2010. The heads were harvested during their development, at 10, 15, 20, and 25 days post anthesis. The grains were immediately separated from the head after harvest, and the embryo were dissected from these grains. Then, both embryos and de-embryonated grains were put in aluminium foil, and frozen immediately in liquid nitrogen. The dissected samples were then stored at -80^{o}C freezer.

The embryo and the de-embryonated grain were ground into a fine powder in liquid nitrogen. The RNA was isolated from this powder by using Spectrum^{TM} Plant Total RNA Kit from Sigma Life Science. The RNA was then stored at -80^{o}C until required.

7.2.1.3 cDNA synthesis

The cDNA was synthesized using Phusion^{TM} RT-PCR Kit from Finnzymes. Up to 1μg RNA were combined with 10mM dNTP mix, 1μL OligodT primer. This mixture was brought to 10μL by adding RNase-free H_2O and then incubated at 65^{o}C for 5 minutes to denature the RNA. The reaction tubes were then placed on ice. Ten times RT buffer (2μL), RT PCR enzyme mix 2 μL and 6μL RNase-free H_2O were added into these reaction tubes. The tubes were then put into Hybaid PCR express thermal cycler, with conditions as follows: primer extention at 25^{o}C for 10 minutes, cDNA synthesis at40^{o}C

for 30 minutes, and reaction termination at 85^{o}C for 5 minutes. The samples were then cooled at 4^{o}C.

7.2.2 Degenerate primer design to amplify CGT and F2H sequences in bread wheat

7.2.2.1 Source of CGT and F2H protein sequences

The sequences used in the alignments for designing the degenerate primers were obtained from various sources. Protein sequences of the *CGT* candidate genes were obtained from Gramene databases (www.gramene.org). Japonica rice sequences (chapter VI) were used as anchor sequences to locate the syntenic locus in *Brachipodium distacyon*, *Sorghum bicolor*, *Zea mays* and Indica rice. The wheat sequence *BJ215997* was obtained through BLAST search facility in Plant expression database website (http://www.plexdb.org/plex.php?database=Wheat).

The homologue F2H and Isoflavone synthases (IFS) protein sequences were obtained from NCBI database (www.ncbi.nlm.nih.gov/) through BLAST search facility. The rice F2H sequence (LOC_Os06g01250) identified in chapter VI was used as query. Other protein sequences of enzymes that work with naringenin as substrate in the flavonoid pathway were obtained from NCBI databases and previous studies by Asenstorfer et al. (2007), Ayabe & Akashi (2006), Cummins et al. (2006), Lepiniec et al. (2006), Martens & Forkmann (1999), Shirley (1998) and Shirley (2001). The unpublished wheat F3H sequences were provided by Dr. Judy Rathjen.

7.2.2.2 Sequence alignments

The multiple sequence alignments of CGT (Figure 7.1) and the F2Hs (Figure 7.2) were performed by MAFFT engine (http://www.ebi.ac.uk/Tools/msa/mafft/) with blossum62 matrix, gap open penalty 1.53 and gap extension penalty 0.123. The alignment was then transferred to GeneDoc version 2.7 (http://www.nrbsc.org/gfx/genedoc/) for further multiple alignments editing, shading and primer design. The percentage identity were analysed by clustalW2.

7.2.2.3 Primer design

Primer pairs for amplification of the *CGT*

Six degenerate primers were designed from the multiple alignments of rice OSCGT and translated protein sequences of the *CGTs* of other cereals and wheat EST *BJ215997* (Figure 7.1). Three of the primers were forward primers (F1, F2, F3) and three were reverse primers (R1, R2, R3) (Table 7.1). Eight pairs of these degenerate primers were tested in the PCR optimisation. Assuming that the CGT protein sequence for wheat is similar to that of rice, *CGT* bands were expected at 1074bp for F1 and R1 primer pairs (CGTF1R1), 1008bp for F2 and R2 primer pairs (CGTF2R2), 1017bp for F1 and R2 primer pairs (CGTF1R2), 1065bp for F2 and R1 primer pairs (CGTF2R1), 564bp for F2 and R3 primer pairs (CGTF2R3), 576bp for F1 and R3 primer pairs (CGTF2R3), and 480bp for F3and R2 primer pairs (CGTF3R2), using cDNA as template.

Primer pairs for amplification of the *F2H*

The conserved regions of F2H and IFS protein sequences were identified by placing these sequences into 2 different groups with different shading colour: the conserved sequences of IFS were highlighted in green, while the contrasts between F2H/FS2

against IFS sequences groups were shaded in purple. The conserved regions of both groups are in black (Figure 7.2). The primers were designed in the sequences with black and purple shading colour, to minimize amplification of *IFS* templates.

Due to time constrains, only the amplification of *CGT* sequence by the degenerate primers was studied. The directions for further experiments in the future to obtain optimum *CGT* amplification methods are proposed.

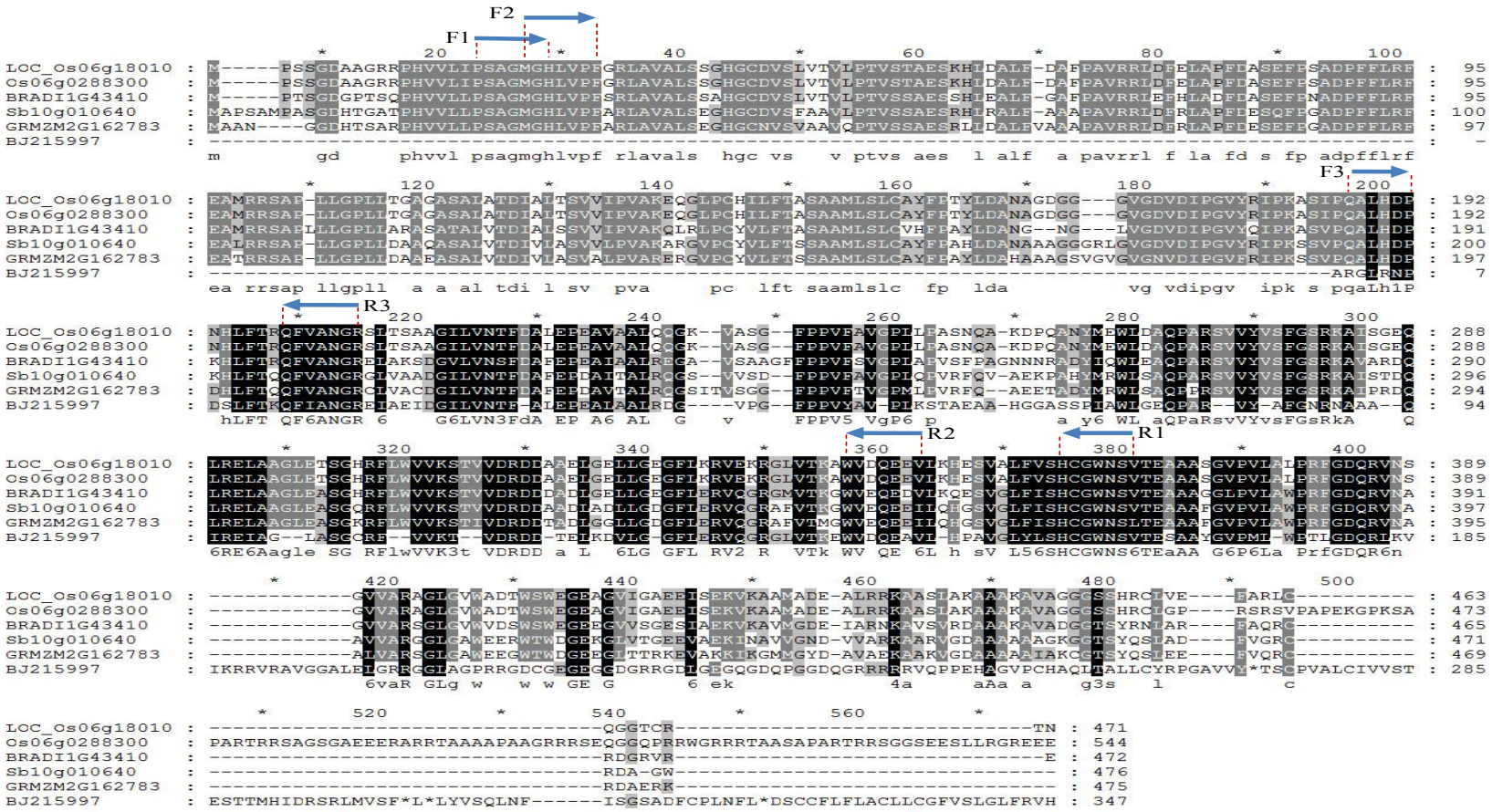


Figure 7.1 Multiple alignments of translated protein of putative *CGT*: LOC_Os06g18010 (Japonica rice OSCGT, Brazier Hicks et al. 2010), Os06g0288300 (Indica rice glycosyltransferase), BRADI1G43410 (*Brachypodium distachyon* glycosyltransferase), Sb10g010640 (*Sorghum bicolor* glycosyltransferase), GRMZM2G162783 (*Zea mays* glycosyltransferase) and BJ215997 (wheat EST homologous to rice *C*-glycosyltransferase). Blue arrows showed the position of the degenerated primers: F1, F2, and F3 are forward primers, while R1, R2 and R3 are reverse primers. The red lines in dash are the start and end of the primer sequences. The grey and black shades showed the sequence conservation.

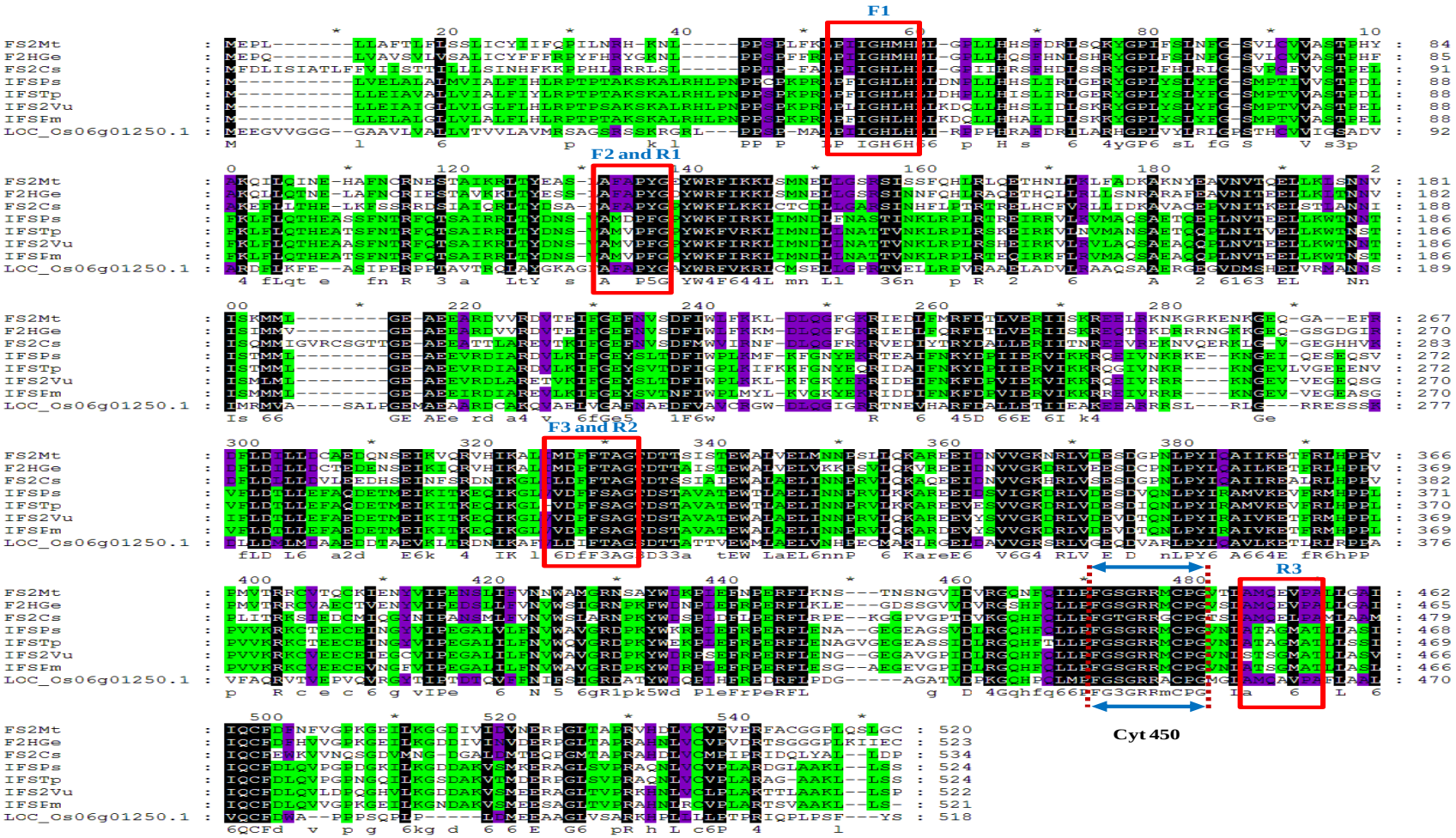


Figure 7.2 Multiple alignments of translated protein of putative *F2H*, *FS2* and *IFS*: LOC_Os06g01250 (Japonica rice F2H, Du et al. 2010), FS2Mt (*Medicago truncatula* F2H, Zhang et al. 2007), F2HGe (*Glycyrrhiza echinata* F2H, Akashi et al. 1998), F2HCs (*Camellia sinensis* FS2 (O. Kintze, unpublished-NCBI

database)), IFSPs (*Pisum sativum* IFS, Cooper 2005), IFSTp (*Trifolium pratense* IFS (Kim et al. 2005, unpublished-NCBI database)), IFS2Vu (*Vigna unguilata* IFS2, Kaur & Murphy 2010), IFSPm (*Pueraria montana* var. lobata IFS, Jeon and Kim (unpublished-NCBI database)). The F2H and IFS sequences are sorted into 2 different groups: the conserved sequences of IFS are highlighted in green, while while the contrasts between F2H/FS2 against IFS sequences groups are shaded in purple. The conserved regions of both groups are in black. Blue arrows show the position of the degenerate primers: F1, F2, and F3 are forward primers, while R1, R2 and R3 are reverse primers. The red boxes are the position of the primers: F1-F3 are forward primers, while R1-R3 are reverse primers. The blue arrow and red lines in dash between 471-481kb are the start and end of the cystein heme-iron ligand signature of the cytochrome P450 protein motif.

Table 7.1 Degenerate Primers to amplify *CGT* in bread wheat

Candidate Genes	No	Amino acid sequence	Reverse (R)/ Forward (F)	Primer sequences
CGT	1	PSAGMGH	F1	CCIWSIGCIGGNATGGGNCA
	2	MGHLVPF	F2	ATGGGICAYYTIGTNCCNTT
	3	PQALHDP	F3	CCICARGCIYTICAYGAYCC
	4	HCGWNSV	R1	GTIACISWRTTCCAICCRCARTG
	5	WVDQEEV	R2	AYIKCYTCYTGITCNACCCA
	6	QFVANGR	R3	CKICCRTTIGCIACRAAYTG

Table 7.2 Degenerate Primers to amplify *F2H* sequences in bread wheat

Candidate Genes	No	Amino acid sequence	Reverse (R)/ Forward (F)	Primer sequences
F2H	1	PIIGHMHM	F1	CCIATHATHGGICAYATGCAYATG
	2	AFAPYG	F2	GCITTYGCICCITAYGGI
	3	MDFFTAG	F3	ATGGAYTTYTTYACICGIGCI
	4	AFAPYG	R1	CCIATYGGICGIAAYCGI
	5	MDFFTAG	R2	CCIGCITGIAAYAAYCTYTAC
	6	MQEVPA	R3	GCI GGI CAY CTR GTR TAC

7.2.3 Amplification of CGT sequence from wheat gDNA and cDNA by the degenerate primer pairs

7.2.3.1 Optimization of PCR

Initially, each degenerate primer pair was used to amplify 1.5 ng/μL gDNA template of Nipponbare rice to optimise the PCR conditions. The first PCR master mix used consisted of 1X PCR buffer, 0.2mM dNTP mixture, 1.5mM $MgCl_2$, 0.2 μM Forward and reverse primers, 5.0 unit Platinum® Taq DNA polymerase (Invitrogen), and water in a total of 50 μL reaction for 6 PCR reactions. The PCR amplification was performed by using Hybaid PCR express thermal cycler with 12 column x 8 rows gradient blocks with samples in every odd block number. The PCR condition was as follows: 94°C for 5 minutes of the first cycle, followed by 40 cycles of the second cycle with 94°C

denaturation for 30 seconds, 30-45^{o}C gradient of annealing temperature range for 30 seconds, and 72^{o}C extension for 2 minutes, and ended the PCR cycle with 72^{o}C for 10 minutes. The amplified fragments were then visualised by gel electrophoresis (100mV, 40 minutes) with 1% Agarose and 0.015μL/mL Invitrogen SYBR® Safe DNA gel stain in 1X TBE buffer (0.09M Tris, 0.09M boric acid, and 2 mM EDTA pH 8.0).

The optimum PCR condition and master mix composition were then used to amplify 8 ng/μL DNA template (gDNA and cDNA) of nullisomic-tetrasomic lines of Chinese Spring group 7, Sunco and Tasman parents, Sunco and Tasman panel lines, water as negative control and the Nipponbare rice as the positive controls. Further optimisations were performed by modification of master mix composition, especially with the types and amount of Taq polymerases; the amount of and $MgCl_2$ and the amount of gDNA and cDNA that were used.

7.2.3.2 Isolation and purification of prospective *CGT* DNA bands

Firstly, the PCR bands obtained with the right size were re-visualised by gel electrophoresis with 1X TAE buffer (0.04 M Tris, 1 mM EDTA pH 8.0). The gel was then viewed over blue light. The visible bands with the expected size were excised by using blade or stabbed by using 200μL pipette tips and then were put into separate tubes.

Secondly, the DNA from the gel was purified by QIAquick® Spin purification Kit. To evaporate the excess ethanol, the purified DNA sample tubes were placed in a 37^{o}C hot block for 10 minutes. The purified DNA obtained was again visualised in 1-1.2% Agarose gel with 0.015mL/mL Invitrogen SYBR® Safe DNA gel stain in TBE buffer,

100mV, 40 minutes. The DNA concentration was measured using Nano-drop spectrophotometer (Thermo Scientific, USA).

7.2.3.3 Cloning and sequencing of prospective CGT DNA bands

Cloning of prospective DNA bands in pDrive cloning vector

The purified DNA bands were cloned in pDrive cloning vector using QIAGEN® PCR cloning Kit. To ligate the purified DNA band with the pDrive plasmid cloning site, 1-4μL of purified PCR products was mixed with 50 ng of pDrive cloning vector and 1X ligation master mix. The mixture was then keep at room temperature (23^{o}C) for 10 minutes and stored at 4^{o}C overnight.

Transformation of p-Drive recombinant plasmid into Escherichia coli

The transformation was performed by electroporation. One μl of ligation product was mixed gently with the *E. coli* competent cells. Then, 60 μL of the mixture was transfered into an electroporation cuvet (BioRad, USA) and incubated on ice for 5 minutes. The electroporation was performed with 1.8V field strength of electrical pulse, capacitance of 25μF, resistance of 200Ohms, and a pulse length of 4-4.5s. Seven hundred μL SOC medium (containing 2% tryptone, 0.5% yeast extract, 10mML NaCl, 2.5 mM KCl, 10mL $MgCl_2$, 10mM $MgSO_4$, and 20mM glucose) was added to electroporated cells and then transferred into a fresh tube and stored at 37^{o}C incubator for 1 hour. The culture was then centrifuged gently at 3500rpm for 2 minutes. The top 500μL supernatant was discarded and the remaining of the culture gently resuspended and poured into Luria broth (LB) media plates containing 0.1 mg/mL ampicilin, 0.03 mg/mL IPTG (isopropylthiogalactoside), and 0.035mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The culture plates were stored at 37^{o}C overnight. The

white colonies were identified and used to inoculate 2mL in LB liquid media containing 0.1 mg/mL ampicilin and incubated at 37^{o}C in a shaker overnight.

DNA Plasmid isolation and purification

The suspension culture (1.5 mL) was transferred to a new tube and centrifuged at 13000rpm for 5 minutes. The supernatant was removed and the DNA plasmids were isolated from the bacterial cells using QIAprep® Miniprep Kit. The tubes containing purified plasmid DNA were then placed in a 37^{o}C hot block for 10-15 minutes to evaporate the excess ethanol.

To check if the PCR product was successfully inserted into the cloning vector, the recombinant plasmids were then cut by EcoRI restriction endonuclease enzyme (Roche). EcoRI enzymes (0.2 units) was mixed with 1X buffer, water and 200 ng of plasmid DNA and incubated at 37^{o}C for 3-12 hours. The restriction products were visualised by gel electrophoresis using 1% agarose with 0.015μL/mL Invitrogen SYBR® Safe DNA gel stain in TBE buffer (100mV, 40 minutes). Two band sizes were expected to be recovered, the pDrive clonning plasmid (3Kb) and the insert PCR product. The positive purified plasmid samples containing PCR product were sent to AGRF for Purified DNA sequencing. The BLASTn and BLASTx search was performed against NCBI databases to identify the function of the protein from the sequencing results.

7.2.4 Amplification of CGT sequence from wheat gDNA and cDNA by nested PCR

To optimise the nested PCR conditions, the wheat gDNA and cDNA were initially amplified by degenerate primer pairs CGTF1R1 and CGTF2R2 (Figure 7.1) with the initial condition as explained in section 7.2.3.1. The results were visualised by gel electrophoresis. The band obtained was excised from the gel and purified as explained in section 7.2.3.2. Next, the second stage of the nested PCR was performed by re-amplifying the initial PCR product by CGTF3R2 degenerate primer pairs. For all these nested PCR amplifications, the rice gDNA and water were used as positive and negative control respectively.

7.2.5 Optimation of PCR for re-amplification of EcoRI restriction product

The inserts of PCR product from amplification by CGTF3R2 primer pairs were recovered from the recombinant plasmids by EcoRI restriction. This sequence was then re-amplified. The re-amplification by PCR was optimised as explained in the earlier sections (7.2.3.1).

7.3 Results and Discussion

7.3.1 Isolation, purification and cloning of CGT candidate genes for ACG ratio

7.3.1.1 PCR optimization

PCR with gDNA

At this stage, amplification of the gDNA with the 8 primers pairs was only successfully optimised with one pair, CGTF3R2. DNA bands with 480kb size were obtained in all of gDNA of nullisomic-tetrasomic Chinese Spring group 7 (figure 7.3 A and B) indicating that the primer was not genome specific.

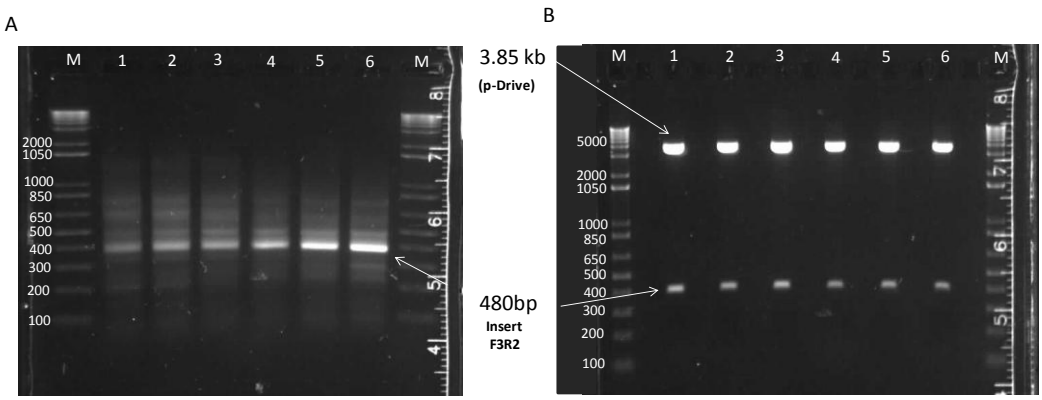


Figure 7.3 A. Results of PCR amplification with primer F3 and R2, DNA of wheat (Chinese Spring) was used as the template. Lane M: 1 kb plus DNA ladder as marker (100-5000 numbers in white are their size); Lane 1-6: bands obtained from 35 extension cycles of PCR with gradient temperature (15^{o}C, every 30 second between 36^{o}C-44^{o}C). B. The results of EcoRI restriction, when the band resulted from amplification with similar primer pairs with different wheat DNA samples of nullisomic-tertrasomic lines of Chinese Spring were cloned in p-Drive cloning vector. The bands at 480bp were the inserts, while the bands at 3.85kb were the p-Drive vectors. Lane M: 1 kb plus DNA ladder as marker. Lane 1: nullisomic 7B tertrasomic 7D lines, lane 2, 4 and 5: 7D7B, 3: 7A7B, 6: 7B7A.

DNA bands with expected size for the product of CGTF3R2 primer pairs (480 kb size) were also recovered in nested PCR (Figure 7.4 B). However the first stage of nested PCR amplification with CGTF1R1 did not show any bands (Figure 7.4 A). It is not clear whether the F1 and R1 primers failed or the amplification was very low. In the case of F1 and R1 primer failure to amplify the gDNA template, the recovery of CGTF3R2 product obtained from the amplification of purified gDNA.

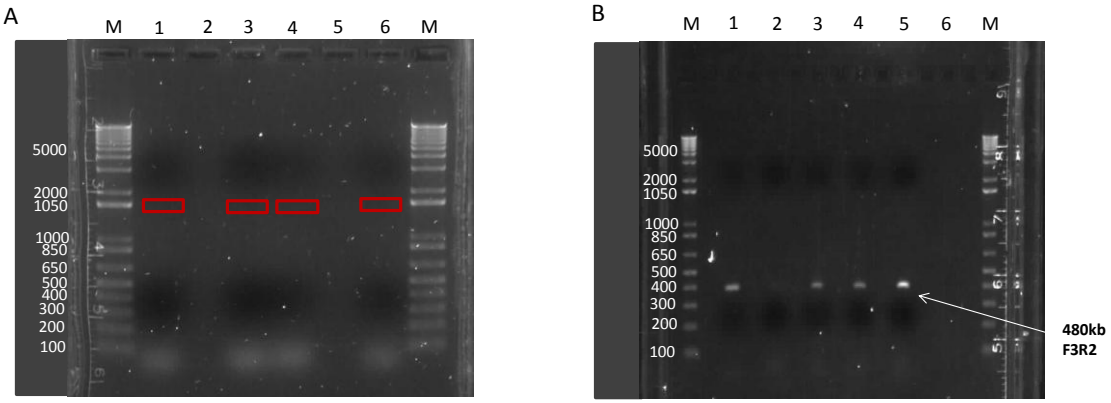


Figure 7.4 Results of Nested PCR amplification: A. First stage of PCR amplification by CGTF1R1 primer pair with Chinese Spring gDNA as template, lane M: 1 kb plus DNA ladder as marker (numbers in white are in kb size), lanes 1, 3, 4 and 6: amplification products, with red rectangles showed the position of the expected amplified bands (1050kb), that were excised prior to purification. B. Second stage of PCR amplification by CGTF3R2 primer pairs with gDNA of Chinese Spring and rice as template, lane M: 1 kb plus DNA ladder as marker (numbers in white are in kb size), lane 1: positive control, the band obtained from amplification of gDNA with CGTF3R2 primer pair without the first stage nested PCR amplification, lane 2 and 3: bands obtained after 2 stages of nested PCR amplification of Chinese Spring gDNA template with CGTF1R1and CGTF3R2 primer pairs, lane 4 and 5: bands obtained after 2 stages of nested PCR amplification of rice gDNA template with CGTF1R1and CGTF3R2 primer pairs.

PCR using a cDNA template

The optimisation of PCR with the cDNA successfully amplified products with CGTF3R2, CGTF2R2 and CGTF1R2 primer pairs (Figure 7.5 A and B). Only one

reverse primer functioned successfully, the R2 primer at the end of C-terminal of the *C*-glycosyltransferase. These results were obtained with the cDNA generated from mRNA of Sunco and Tasman embryos, Sunco seed coat and gDNA of Chinese Spring wheat and rice.

Amplification by the degenerate primers with cDNA but not with the gDNA might indicate that optimum PCR conditions have not been met for PCR with the gDNA. Alternatively, there might be differences in the exon-intron arrangements between rice and wheat. There is no intron in the rice *CGT* sequence, but this does not necessarily apply to the gene in wheat. In addition, this might also be caused by the high sequence copy numbers in the wheat genome and high variation in the gDNA, that codes for glycosyltransferase sequences.

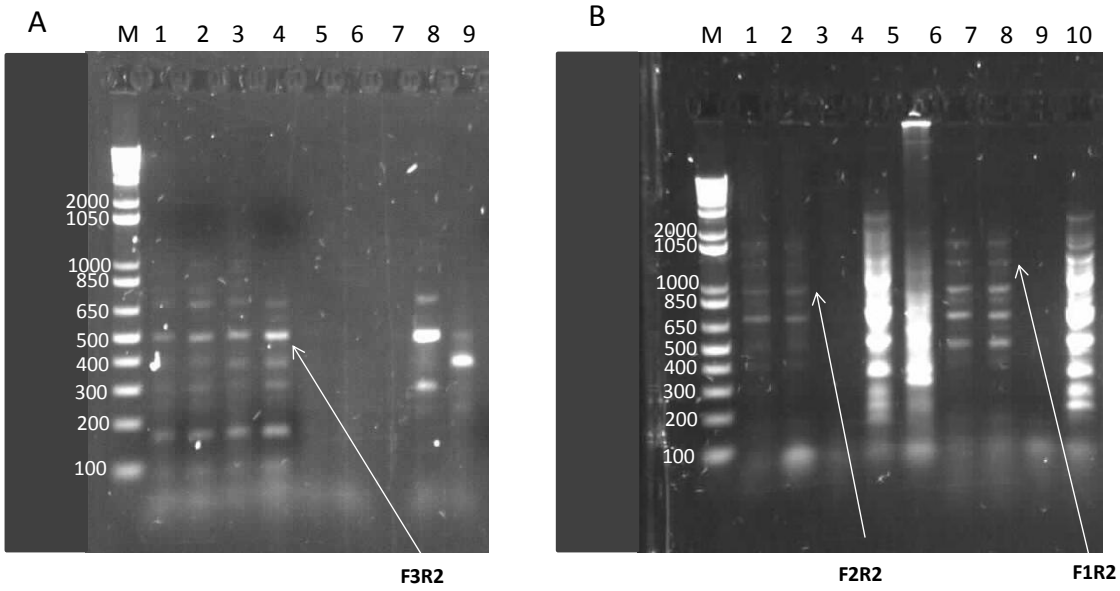


Figure 7.5 Results of PCR optimisation for CGTF3R2, CGTF2R2 and CGTF1R2 primer pairs with cDNA as template. The cDNA were constructed from mRNA isolated from embryo and seed coat of Sunco and Tasman parents. A. PCR optimized for CGTF3R2 primer pair. cDNA templates: 1,2: Sunco embryo; 3-4: Tasman embryo; 5-6: Sunco seed coat; 7 and 8 were rice and Chinese Spring wheat as positive controls respectively; 9: water as negative control B. PCR optimized for CGTF2R2 (2-6, 11, 12) and CGTF1R2 (7-10, 13, 14) primer pairs. cDNA templates: 2, 7: embryo of Sunco; 3, 8: embryo of Tasman; 4, 9: seed coat of Sunco. Rice (5 and 10) and Chinese Spring DNA (6) were used as positive controls. M: DNA ladder marked with kb in white.

Re-amplification of PCR products of CGTF3R2 primer pairs with cDNA template from Sunco and Tasman embryo

To recover more products for further analyses, PCR amplification was repeated with F3R2 following EcoRI restrictions. The results are shown in figure 7.6. Very faint bands were recovered.

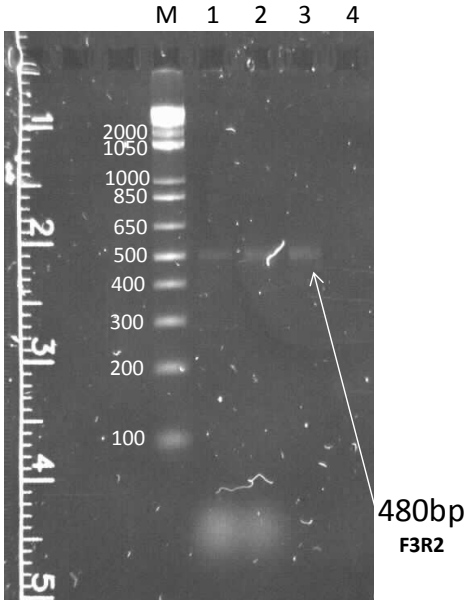


Figure 7.6 Results of re-amplification of PCR products of CGTF3R2 primer pairs with cDNA template of mRNA from embryo of Sunco (1, 2) and Tasman (3, 4). M: DNA ladder marked with kb in white).

7.3.1.4 Sequences obtained from the amplification

The purified DNA sequencing results of PCR amplification by gDNA and cDNA using F3R2 primer pairs were shown in Figure 7.7, 7.8 and 7.9. The translated protein and DNA sequences from this amplification are similar for all clones. However, none these sequences (protein and DNA) corresponded to *CGT*; except for the forward (F3) and reverse (R2) degenerate primer sequences. The F3 primer sequences (Table 7.1) were

recovered from the 1st 5‟-3‟ frame translation (Figure 7.8), while the R2 sequences (Table 7.2) were recovered from the 2nd 5‟-3‟ frame translation (Figure 7.9).

The shift in the 5‟-3‟ frame translation of wheat sequences were also observed with *BJ215997* EST sequence translation that was used in the designing the degenerate primers. This EST sequence was short and only covers half length of the CGT sequence. Error and unidentified peaks in the previous sequencing report might cause this translation shift.

The results of BLAST search of the translated protein and DNA sequences of CGTF3R2 amplification recovered sequences of transposons in wheat 3B chromosome BAC library (92-94% similarity, covered 83-93% coverage of query sequences) (Table 7.3). Several coding sequences of this transposable element had putative glycosyltransferase like functions in *Brachypodium distachyon* (Table 7.3). However, the similarity between this putative glycosyltransferase HGA-like and *C*-glycosyltransferase DNA sequences are low, only ranged between 44-67% of sequence identity which covers 85-100% of the query sequences.

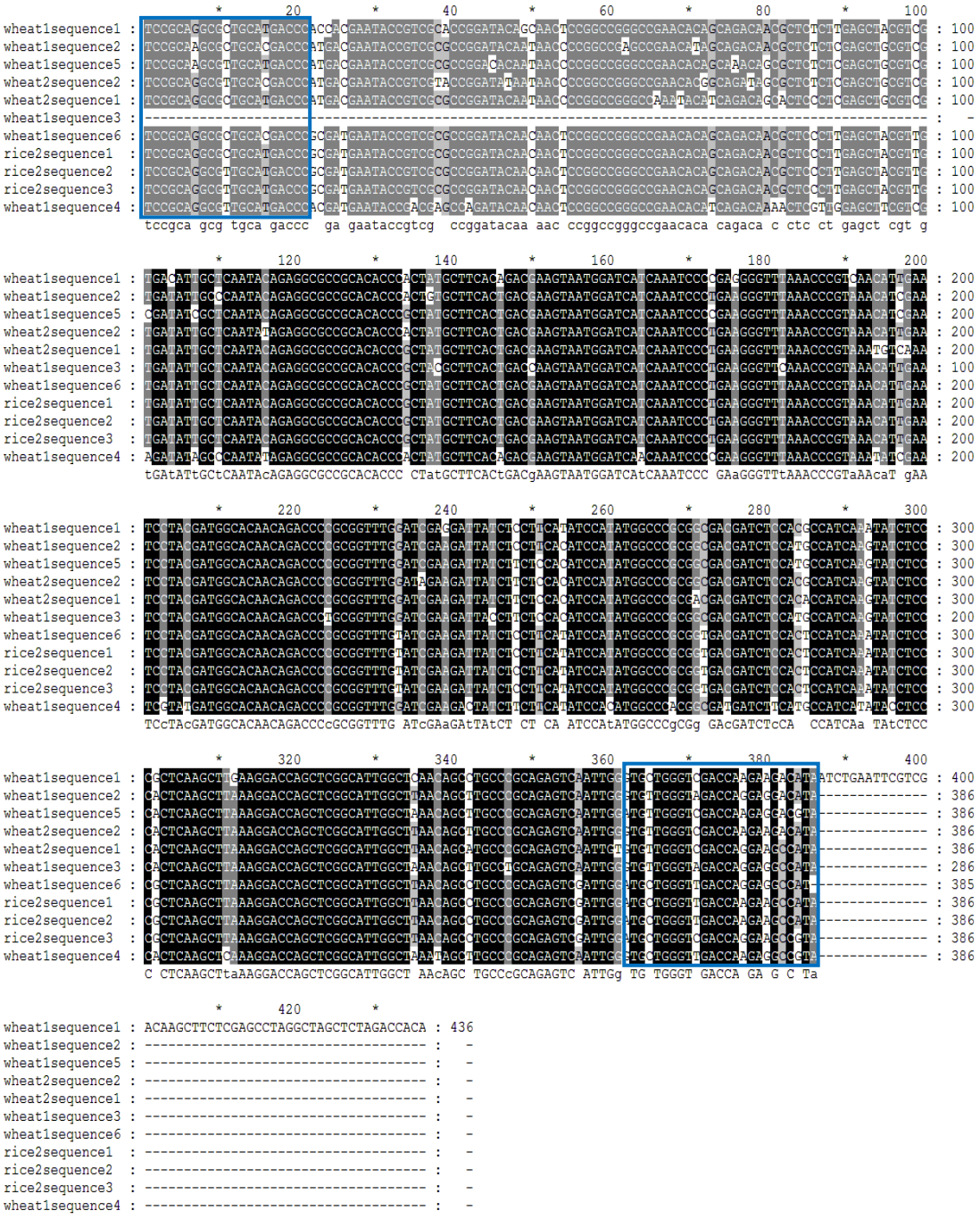


Figure 7.7 Multiple alignments of DNA sequences, obtained from PCR amplification with genomic DNA template (sequences coded wheat1) and cDNA template (sequences coded wheat2 and rice2). Sequence codes: sequence1, sequence2, sequence3, sequence4, sequence5 and sequence6 indicated the identification of the *E.coli* clones. The sequences in the blue rectangle show the position of the F3 and R2 primer in each sequence. The grey and black shades show the sequence conservation. Alignment was performed by MAFFT online alignment tools in http://www.ebi.ac.uk.

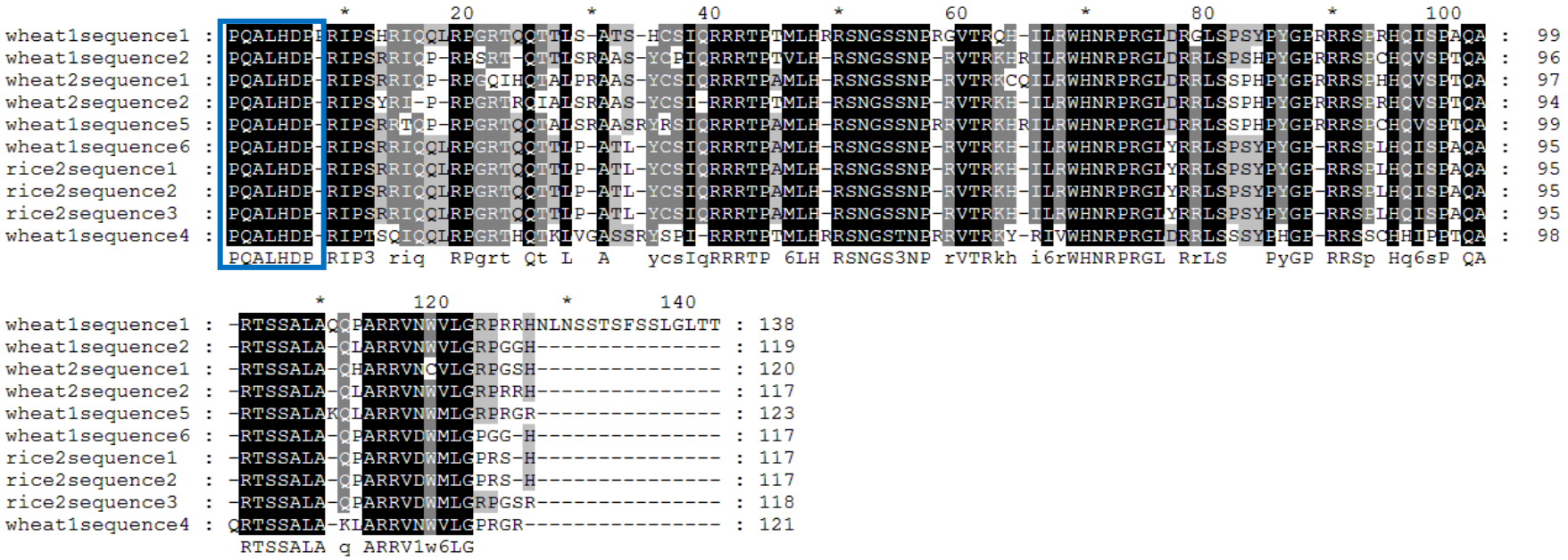


Figure 7.8 Multiple alignment of translated protein sequences, which nucleotides obtained from PCR amplification with gDNA template (sequences coded wheat1) and cDNA template (sequences coded wheat2 and rice2). Sequence codes: sequence1, sequence2, sequence3, sequence4, sequence5 and sequence6 indicated the identification of the *E.coli* clones. The amino acid sequences in the blue rectangle show the position of the F3 primer in each sequence. The grey and black shades show the sequence conservation. Alignment was performed by MAFFT online alignment tools in http://www.ebi.ac.uk.

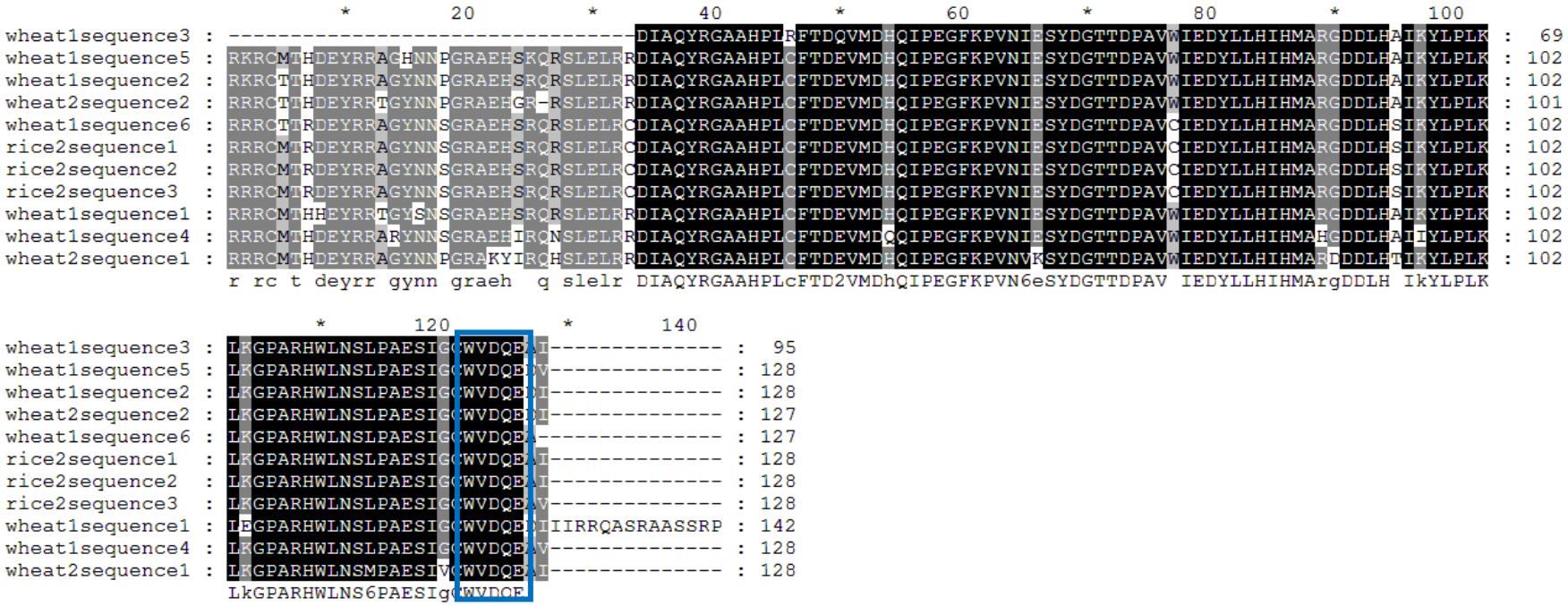


Figure 7.9 Multiple alignment of translated protein sequences, which nucleotides obtained from PCR amplification with gDNA template (sequences coded wheat1) and cDNA template (sequences coded wheat2 and rice2). Sequence codes: sequence1, sequence2, sequence3, sequence4, sequence5 and sequence6 indicated the identification of the *E.coli* clones. The amino acid sequences in the blue rectangle show the position of the R2 primer in each sequence. The grey and black shades show the sequence conservation. Alignment was performed by MAFFT online alignment tools in http://www.ebi.ac.uk.

Table 7.3 Results of megablastn into nucleotide collection. DNA sequences (figure 7.4) were used as query sequences.

Genebank accession	Contig	Similarity (%)	Coverage (%)	Plants	Putative function Locus	Function	Similarity(%)	Coverage (%)
FN564432.1	*Triticum aestivum* chromosome 3B-specific BAC library, contig ctg0616b	94	86					
AM932686.1	*Triticum aestivum* 3B chromosome, clone BAC TA3B95G2	93	93					
FN564434.1	*Triticum aestivum* chromosome 3B-specific BAC library, contig ctg0954b	92	83	*Brachypodium distacyon*	Bradi3g28010.1	xyloglucan galactosyltransferase	61	97
					Bradi2g01350.1	fructose-bisphosphate aldolase	94	100
					Bradi2g25170.1	glutathione S-transferase, putative	73	97
					Bradi2g01370.1	phosphatidylserine synthase	96	97
					Bradi2g01380.1	glycosyltransferase, HGA-like	67	100
					Bradi2g01390.1	glycosyltransferase, HGA-like	73	100
					Bradi3g37830.1	glutamate decarboxylase	87	100
					Bradi2g01420.1	glycosyltransferase, HGA-like	59	100
					Bradi2g01420.1	glycosyltransferase, HGA-like	64	100
					Bradi2g01480.1	glycosyltransferase, HGA-like	66	100
					Bradi4g00290.1	sodium/hydrogen exchanger	82	100
					Bradi2g14950.1	anaphase-promoting complex subuni	93	85
					Bradi2g25450.1	glycosyltransferase, HGA-like	44	86
					Bradi2g40020.1	exonuclease	57	100
					Bradi2g01510.1	alanyl-tRNA synthetase	83	100
					Bradi1g24340.1	cytochrome P450	74	99

7.3.2 Proposed Future work

7.3.2.1 Identification of more Wheat EST sequences

More wheat ESTs (Table 7.4) were obtained from various sources. One EST was obtained through BLAST search facility in Plant expression database website (http://www.plexdb.org/plex.php?database=Wheat), two EST sequences were obtained through BLAST searches in NCBI databases, while the other two sequences were obtained from wheat GlycosylTransferase Inventory database (GTIdB, http://wwwappli.nantes.inra.fr:8180/GTIDB/). The exon sequence of *LOC_Os06g18010* was used as query for these searches. The predicted protein sequences from these wheat ESTs were obtained through ExPASy DNA/RNA translation engine (http://web.expasy.org/translate/).

7.3.5.2 Putative function of the wheat EST sequences

Predicted protein sequences of 4 more wheat ESTs were aligned with amino acid sequence of the rice CGT. They are similar to the rice sequence with 39-62% ID (Table 7.5). These proteins putatively function as CGT, but they are short, only 219-278 amino acids, in comparison to that of rice (471 amino acids). Two sequences, CA699411 and CV773251 were similar to the N-terminal of the CGT (Figure 7.10). The other 2 sequences, CJ606538 and CJ711440, aligned well with the C-terminal of the rice CGT (Figure 7.11).

Table 7.4 Information on the wheat ESTs that were used to design primers for targeting CGT sequence(s) in bread wheat.

Source	DbEST id	EST name	Gene bank Accession	Plant TA accession	Clone Info	Wheat cultivar	Tissue
PLexdB	11993314	BJ215997	BJ215997	BJ215997	wh9f13 (3')	Chinese Spring	spike at
NCBI	26355865	FGAS067647	CV773251	CV773252	WEF077 Row F column 19	Norstar	Crown and
NCBI	15514241	wlk8.pk0017.g5	CA699411	CA699412	wlk8.pk0017.g5 (5' end)	Stephens	leaf
TIGR/GTIdB	38843062	CJ711440	CJ711440	TA105202_4565	whv3n8j04 (5')	Valuevskaya	shoot
TIGR/GTIdB	38786813	CJ606358	CJ606358	TA105202_4565	rwhv3n8j04 (3')	Valuevskaya	shoot

Table 7.5 Similarity between wheat ESTs that were used to design primers for targeting CGT sequence(s) in bread wheat and rice CGTs.

Sequence	Length	Sequence 2	Length	Score
BJ215997	395	LOC_Os06g18010	471	39
CA699411	219	LOC_Os06g18010	471	43
CJ606358	230	LOC_Os06g18010	471	62
CJ711440	278	LOC_Os06g18010	471	62
CV773251	272	LOC_Os06g18010	471	57

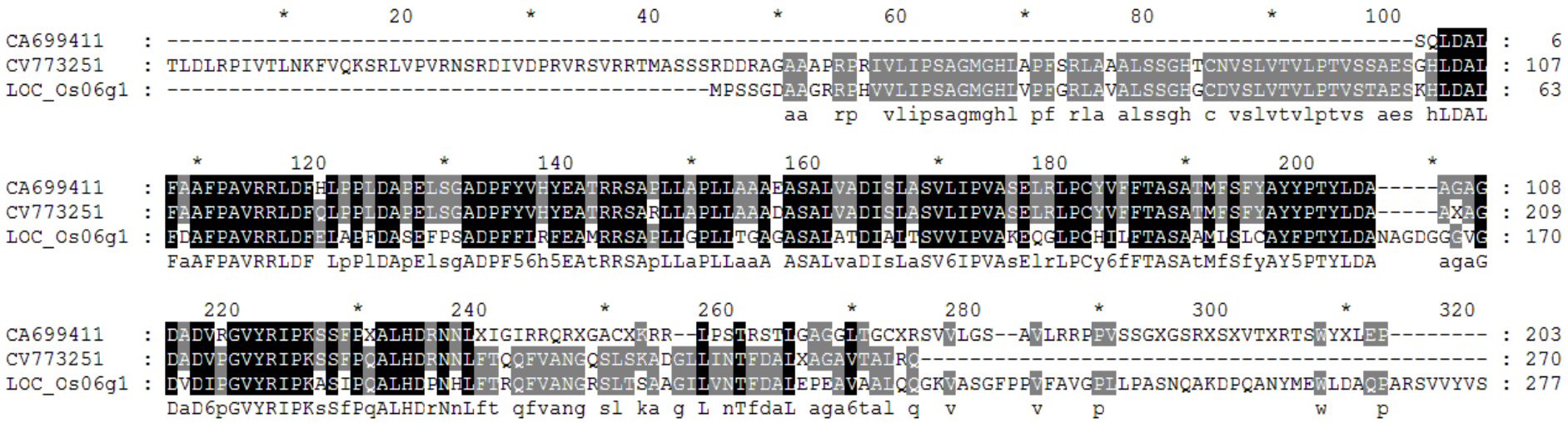


Figure 7.10 Alignment of protein sequences of wheat ESTs CA699411and CV773251 that have similarity to the *N*-terminal domain of rice CGT.

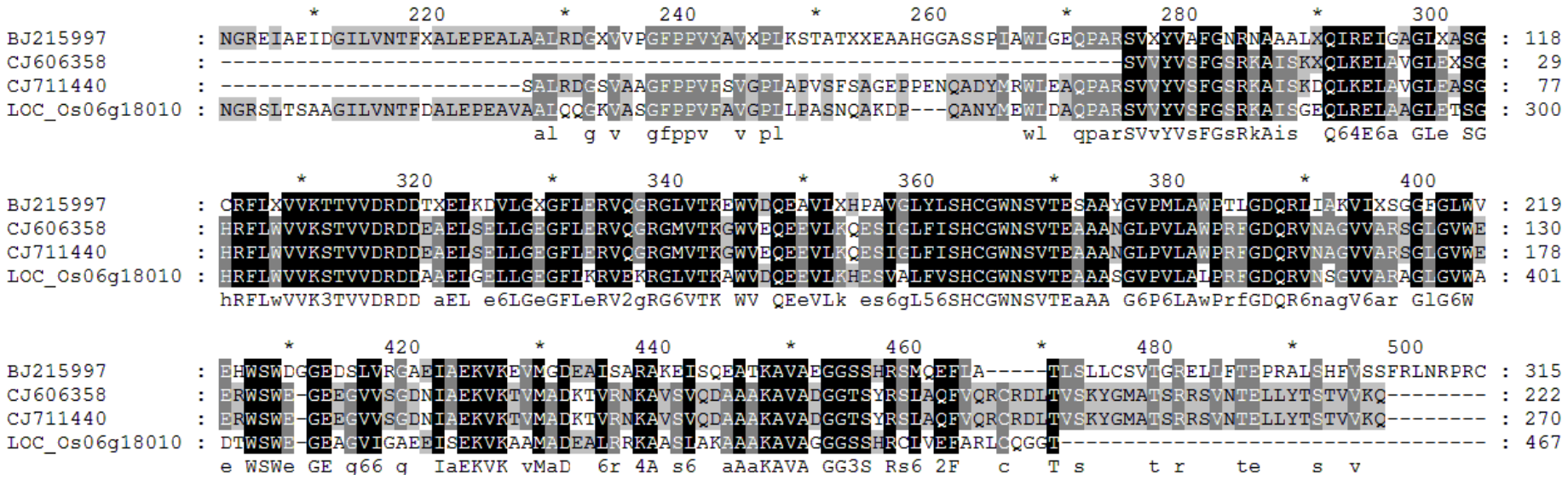


Figure 7.11 Alignment of protein sequences of wheat ESTs BJ215997, CJ606358, and CJ711440 that have similarity to the *C*-terminal domain of rice CGT.

7.3.5.3 Rapid amplification cDNA ends (RACE)-PCR or chromosome/subgenome walking to obtain the full sequence of CGT from wheat ESTs

The EST sequences are short and only covered the exons. It requires other methods such as RACE-PCR and/or genome walking to obtain the full genomic sequences of the EST.

RACE-PCR (Life Technology™ Protocol-Invitogen)

Firstly, specific degenerate primers could be designed to amplify each EST (at least to anneal at 300bp from the 5‟ end) and the PCR conditions to amplify these ESTs from the cDNA with the designed primer need optimised. This could be initiated by methods described in section 7.2.3. These primers could then be used to synthesize cDNA prior to purification and tailing at the 3'-end of the cDNA. From the sequence recovered, another nested primer located within the cDNA could be designed to obtain the full sequence of the EST, either towards the 5‟-3‟ prime end or towards 3‟-5‟ prime end, and RACE-PCR performed.

Chromosome/subgenome walking

The application of the genome walking protocols is very difficult with bread wheat due to the complexities of its genome. The large size of hexaploid wheat genome with high numbers of repetitive sequences, make it is very difficult to walk for a quite large genomic fragment, up to 1 megabse, while restricting it to a specific genome region (chromosome) (Feuillet et al. 2003). This method would require a high resolution map, and a large insert library, which has not yet completed for all

hexaploid chromosomes of bread wheat (Stein et al. 2000, Yan et al. 2003). However, modification of genome walking in a subgenome (A, B or D) of diploid *Triticum* and Aegilops wheats have been successful to obtain various genes (Faricelli et al. 2010, Feuillet et al. 2003, Stein et al. 2000, Yan et al. 2003)**.** To apply subgenome walking to recover the candidate gene sequence of 7BS QTL for ACG traits, require diploid wheat BAC library with 7B chromosome from *Aegilops speltoides*. To avoid the chromosome walking being stopped by high number of repeated sequences, BAC of rice and *Brachypodium* should also be used at the same time (Faricelli et al. 2010, Feuillet et al. 2003, Stein et al. 2000, Yan et al. 2003). In addition, specific probe or PCR primers for the candidate genes will also be required to screen the BAC library.

Specific primers and optimum PCR conditions for amplification of the candidate genes or ESTs with the CGT candidate genes putative function, and/or for markers flanking 7BS QTL locus is very crucial for the application of PCR based genome walking methods. Having highly specific primer pairs and optimum PCR conditions for the candidate genes of carotenoid biosynthesis, Howitt et al. (2009) successfully isolated the candidate gene sequences with PCR based genome walking method from hexaploid bread wheat cultivar Chinese spring.

7.4 Conclusion

The preliminary trial of PCR based methods to amplify the *CGT* genes from genomic DNA and cDNA of bread wheat faced significant challenges due to the high variation in glycosyltransferase sequences in plants as well as the complexity in

wheat genome due to its large size and high number of repetitive sequences. Although sequences with putative glycosyltransferase like function were identified, this experiment is still in-conclusive because of these challenges. Further optimization of the PCR with the existing degenerate primers for the genes and ESTs should be continued. After the specific primers and optimum PCR conditions are optimised, the experiment could be continued with RACE-PCR and subgenome walking to obtain the full sequence of the candidate genes. Similar process could also be applied to *F2H*.

Chapter VIII: General Discussion

8.1 Proposed genetic control of ACG biosynthesis in bread wheat grain

The strong correlation between amounts of ACG1, ACG2 and total ACG, and the lack of significant correlation between ACG content and ACG1/ACG2 suggests that genetic mechanisms that control total ACG content act prior to the glycosylation steps in biosynthesis. Variation in ACG content could reflect the relative activity of the enzymes located early in the flavonoid pathway, competition with other branches of the pathway for flavone precursors and/or UDP sugars, or possibly regulation of flavonol-2-hydroxylase, the first committed step in the proposed branch in the flavonoid biosynthesis pathway leading to ACG. A lower content was previously associated with lower activity of enzymes such as chalcone isomerase, an enzyme catalyzing an early step in flavonoid biosynthesis (Kashem & Mares 2002).

Variation in the ACG ratio provides a means for investigating on the mechanisms and regulation of *C*-glycosylation. No ACGs containing the same sugar residue at both C6 and C8 were identified and each ACG isomer contained arabinose attached to either of these carbons. Since changing the ratio did not affect total ACG content, it seems possible that the proportions of ACG1 and ACG2 depended on either the relative sizes of the available pools of UDP-glucose and UDP-galactose, the activity of the epimerase that catalyses their inter-conversion or variation in the activity and specificity of the *C*-glycosyltransferases. Different alleles of genes coding for *C*-hexosyltransferase may result in differences in UDP-sugar recognition.

Elimination of chromosome 7B in Chinese Spring 7B nullisomic lines resulted in an increase in ACG1/ACG2, i.e. a relative increase in the glucose-containing isomer, possibly indicating the presence of a *C*-glycosyltransferase on 7B with specificity for UDP-galactose. A similar phenotype in the high ratio cultivars could be explained by a deletion or mutation of a gene controlling this enzyme.

Major QTL were detected at 7BS, whilst minor QTL which co-located with QTL for grain size were detected at 4B. The 7BS QTL was identified at the same position after the fine mapping. Alignment of wheat 7BS with the physical maps of rice and *Brachypodium distacyon* was used to identify potential candidate genes that could be involved in control of ACG composition included: a sugar transporter that could determine the relative sizes of the available pools of UDP-glucose and UDP-galactose, an epimerase that might function in inter-conversion of these sugars and glycosyltransferases possibly invovled in the UDP-hexose sugar transfer to the A flavone ring. In addition, a F2H that might control the total ACG was also identified on the same chromosome. The model of genetic control based on these finding and showing possible homologous genes from rice, is proposed in Figure 8.1.

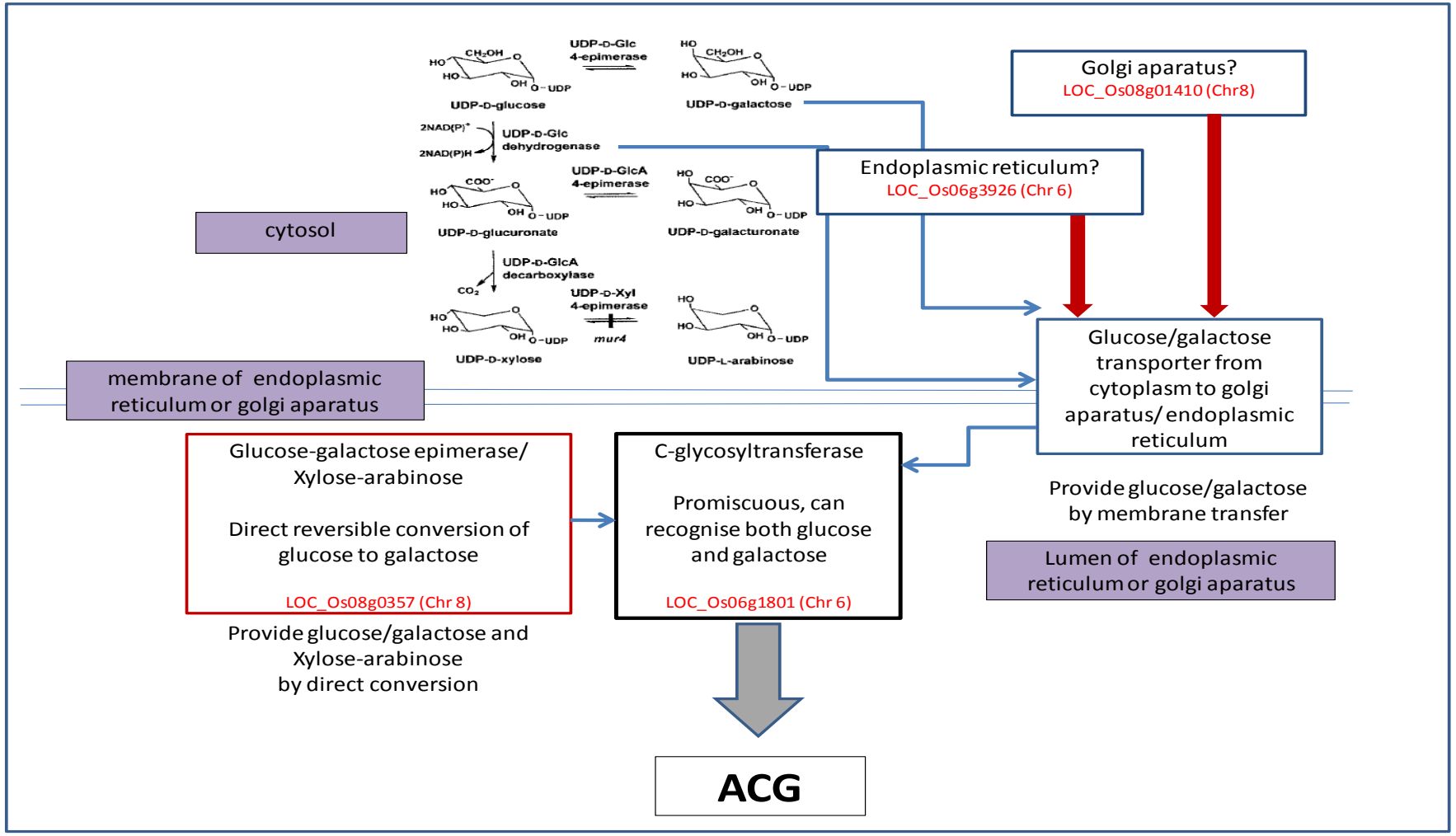


Figure 8.1 Proposed model for ACG biosynthesis in bread wheat grain. The rice genes are shown in red.

8.2 Implication of the proposed genetic control for future study and application of the ACGs

ACGs contribute to yellow colour of YAN in an additive manner with lutein. In comparison with lutein, the ACGs are present in higher concentration but have lower coefficient of extinction. Increasing concentration could potentially increase YAN b* without impacting on use for other end products and reduce the need for colour additives in some commercial products. However, the level of genetic variation showed in this study is not sufficient to achieve the yellowness of commercial noodles and there is also no significant correlation between total ACG content and yellowness of YAN (b*) due to the low recovery of ACGs in the flour after milling.

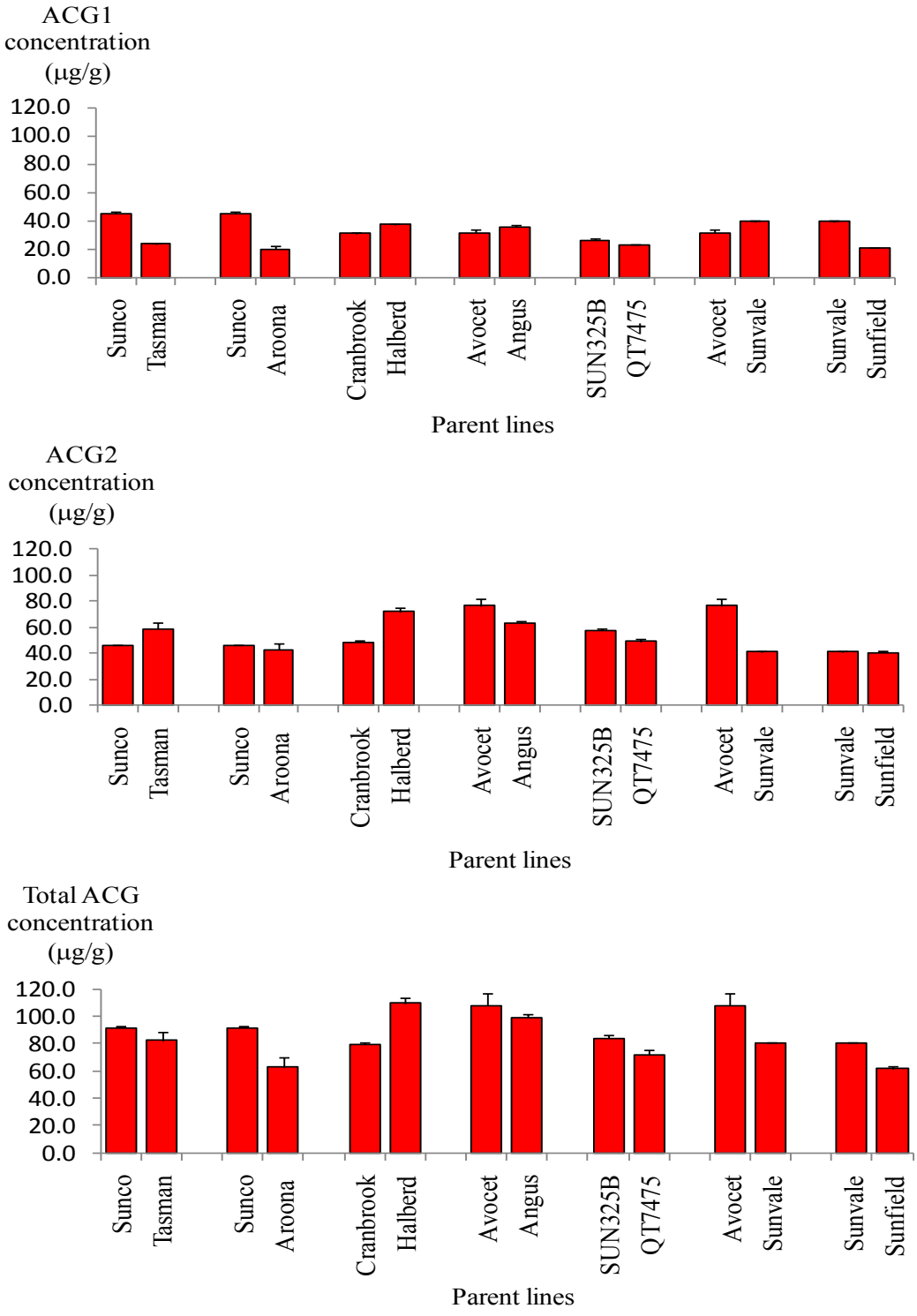
The recovery of the ACGs in the flour after milling was influenced by the physical properties of the grain and their location in outer parts of the grain (bran and pollard tissue) which are discarded during milling. The removal of these parts from endosperm during milling is influenced by the grain softness/hardness and moisture pre-conditioned before milling. This was also confirmed in the results of experiment with the F1 generations of Sunco/Tasman and Gamut/Reeves when the grains were dissected and the ACG were analyses from different tissues obtained. No ACG has been found in the endosperm.

Future options for increasing ACG concentration for YAN might include: incorporation of embryo to the flour after milling and genetic modification of bread wheat to achieve ACG expression in the starchy-endosperm. As the ACGs have also been reported as feeding resistant for brown plant hopper in rice cropping system,

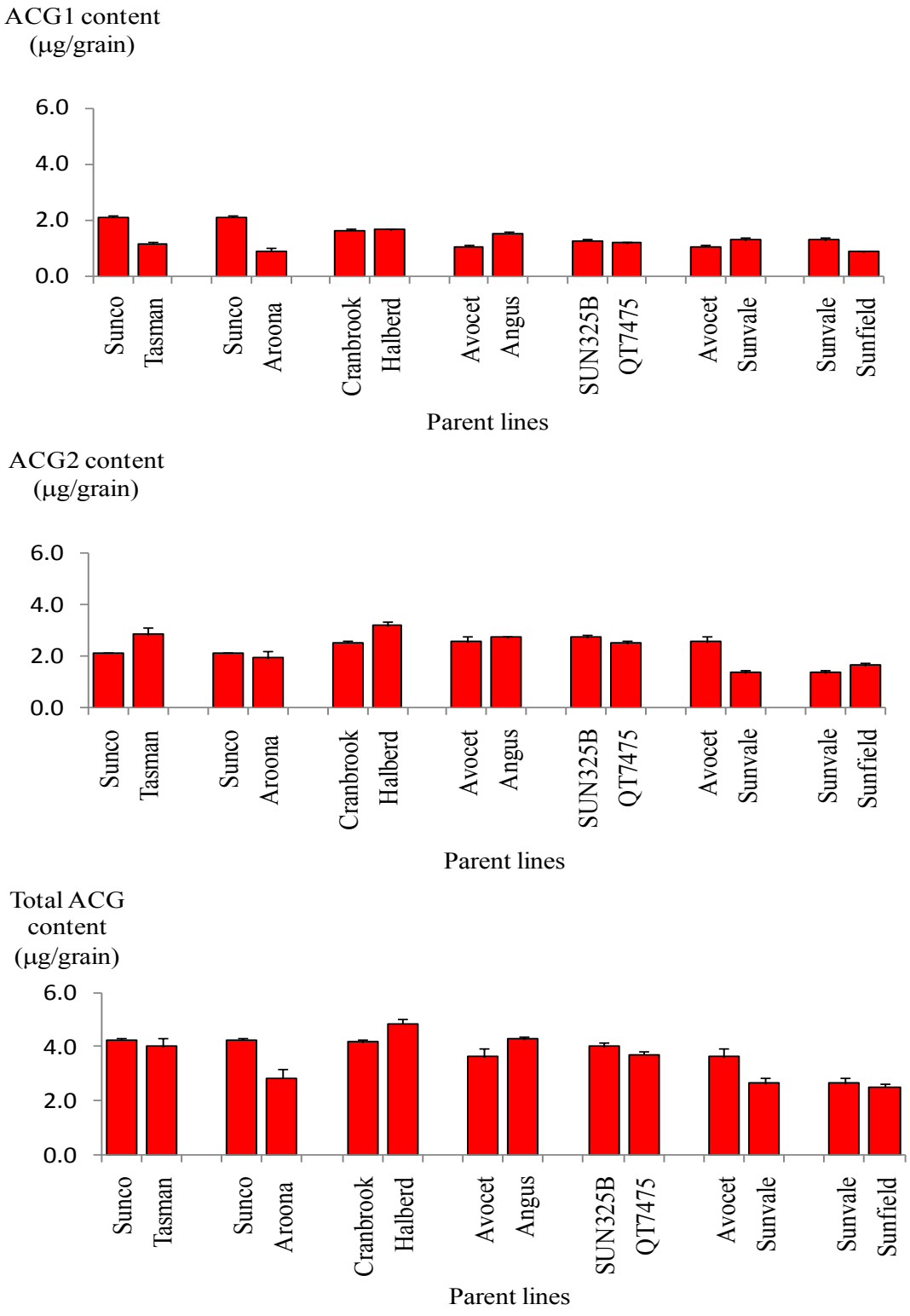
and the candidate genes identified, they also can be studied in rice to developed brown plant hopper resistant rice varieties through breeding and biotechnology.

Supplemental materials

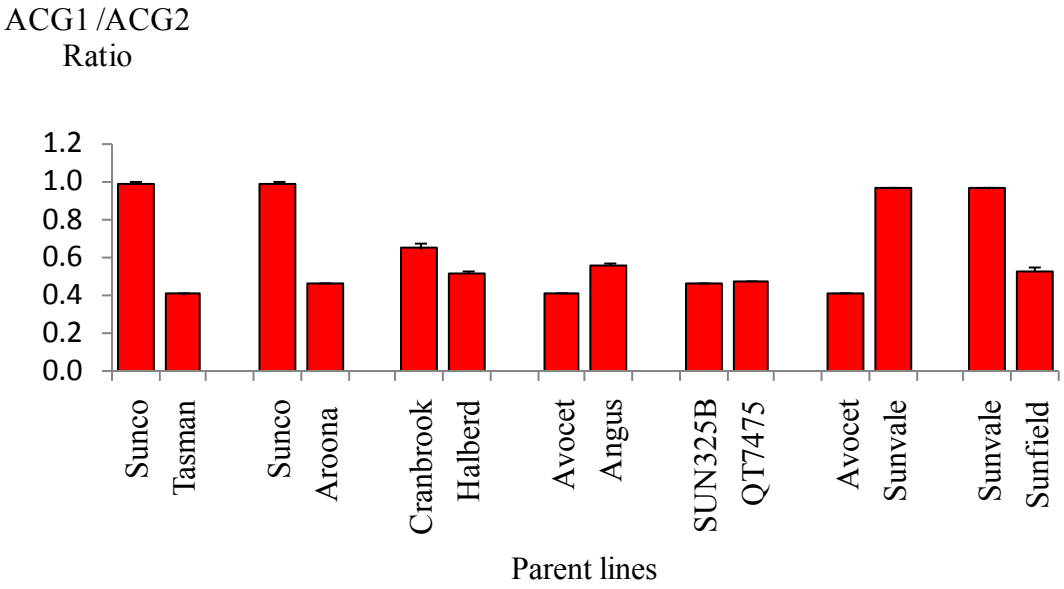
Supplemental material 3.1 ACG concentration (μg/g) (analysed from the grains) for parents of existing doubled haploid populations. Bars represent SE of means. From the top to the bottom: ACG1 concentration, ACG2 concentration and Total ACG concentration



Supplemental material 3.2 ACG content in the grain (µg/grain) of parents of existing doubled haploid populations. Bars represent SE of means. From the top to the bottom: ACG1 content, ACG2 content and total ACG content.



Supplemental material 3.3 ACG composition (ACG1/ACG2 ratio) in grain of parents of existing doubled haploid populations. Bars represent SE of means.



Supplemental material 3.4 Layout of Sunco/Tasman doubled haploid population trial at Narrabri, New South Wales, Australia. The Sunco and Tasman parent lines were planted alternately, as one plot for every 20 plots of doubled haploid lines. Information on this layout was provided by Assoc. Prof. Daryl Mares.

A. Layout for the old lines (147 lines on the revised genome map).

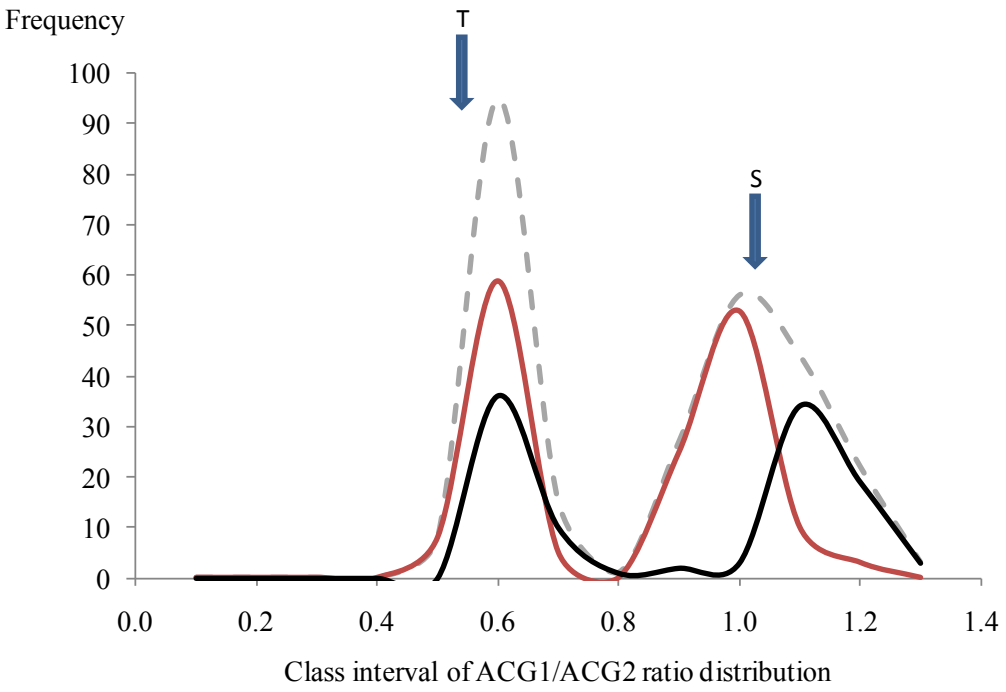
Sunelg	Sunelg	Sunelg	Sunelg	Sunelg	Sunelg	Sunelg	173	172	171	170	169	168	167	166	165	164	163
145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	Tasman	162
144	143	142	Sunco	140	139	138	137	136	135	134	133	132	131	130	129	128	127
109	110	111	112	113	114	115	116	117	118	119	120	Tasman	122	123	124	125	126
108	107	106	105	104	103	102	Sunco	100	99	98	97	96	95	94	93	92	91
73	74	75	76	77	78	79	80	Tasman	82	83	84	85	86	87	88	89	90
72	71	70	69	68	67	66	65	64	63	62	Sunco	60	59	58	57	56	55
37	38	39	40	Tasman	42	43	44	45	46	47	48	49	50	51	52	53	54
36	35	34	33	32	31	30	29	28	27	26	25	24	23	22	Sunco	20	19
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

Supplemental material 3.4 Layout of Sunco/Tasman doubled haploid population trial at Narrabri, New South Wales, Australia (continued)

B. Layout for the new lines (108 lines additional lines for the fine mapping)

Sunelg	Sunelg	Sunelg	Sunelg	Sunelg	Sunelg	314	313	312	311	310	309
297	298	299	300	301	302	303	304	305	306	307	308
296	295	294	293	292	291	290	289	288	287	286	285
273	274	275	276	277	278	279	280	Tasman	282	283	284
272	271	270	269	268	267	266	265	264	263	262	Sunco
249	250	251	252	253	254	255	256	257	258	259	260
248	247	246	245	244	243	242	Tasman	240	239	238	237
225	226	227	228	229	230	231	232	233	234	235	236
224	223	222	Sunco	220	219	218	217	216	215	214	213
201	202	203	204	205	206	207	208	209	210	211	212

Supplemental material 3.5 The frequency distribution of ACG1/ACG2 ratio in 2 sets of Sunco/Tasman doubled haploids. The black lines represent the old population, the red lines represent additional lines, and the dashed grey lines represent the distribution of the whole data set. The blue arrows indicate the means of the ratio trait in the parents (T: Tasman, S: Sunco).



Supplemental material 4.1 List of germplasm and its origin used in this study together with data for ACG1, ACG2 and Total ACG content (μg/g and μg/grain); and ACG1/ACG2 ratio.

No	Cultivars	Origin	ACG1 (μg/g	ACG2 (μg/g	Total ACG (μg/g	ACG1 (μg/grain)	ACG2 (μg/grain)	Total ACG (μg/grain)	ACG1/ACG2 Ratio
I	*Triticum aestivum* Cultivars from Australian								
1	Cunningham	Queensland	29.5	62.7	92.2	1.2	2.6	3.9	0.5
2	EGA Gregory	Queensland	15.7	37.3	53.0	0.8	1.8	2.6	0.4
3	Hartog	Queensland	22.6	53.2	75.8	0.9	2.1	2.9	0.4
4	Janz	Queensland	30.3	41.9	72.2	1.3	1.8	3.1	0.7
5	EGA Hume	Queensland	21.5	46.2	67.7	1.0	2.1	3.1	0.5
6	Kennedy	Queensland	14.8	44.7	59.6	0.7	2.2	3.0	0.3
7	Lang	Queensland	33.8	70.9	104.7	1.5	3.1	4.6	0.5
8	Batavia	Queensland	16.1	38.5	54.5	0.7	1.7	2.5	0.4
9	Baxter	Queensland	27.7	35.4	63.1	1.2	1.5	2.7	0.8
10	Spica	Queensland	20.5	41.1	61.6	1.3	2.5	3.8	0.5
11	Tasman	Queensland	23.7	58.8	82.5	1.1	2.9	4.0	0.4
12	Cook	Queensland	33.3	32.0	65.3	1.6	1.5	3.1	1.0
13	Ventura	Northern NSW	19.3	51.6	70.9	0.9	2.3	3.2	0.4
14	Sunvale	Northern NSW	39.6	41.2	80.7	1.3	1.4	2.7	1.0
15	Sunco	Northern NSW	45.4	45.9	91.3	2.1	2.1	4.2	1.0
16	Sunlin	Northern NSW	27.3	50.5	77.8	1.2	2.2	3.4	0.6
17	Gamut	Northern NSW	69.8	59.7	129.5	3.0	2.6	5.6	1.2
18	Yellow Kite	Northern NSW	32.1	56.6	88.7	1.3	2.3	3.5	0.6
19	Sunfield	Northern NSW	21.2	40.4	61.6	0.9	1.6	2.5	0.5
20	Gatcher	Northern NSW	31.3	32.8	64.0	1.6	1.7	3.4	1.0
21	Suneca	Northern NSW	24.4	48.2	72.6	1.2	2.4	3.6	0.5
22	Gabo	Northern NSW	60.7	58.3	119.0	2.7	2.6	5.4	1.0
23	Condor 17	University of Sydney	50.1	44.5	94.6	2.2	1.9	4.1	1.1
24	Condor 19	University of Sydney	22.0	33.8	55.8	0.7	1.1	1.8	0.7
25	Diamondbird	Southern NSW	13.0	38.2	51.2	0.6	1.7	2.2	0.3
26	Avocet	Southern NSW	31.4	76.4	107.8	1.1	2.6	3.6	0.4
27	Chara	Victoria	17.5	36.9	54.4	0.8	1.7	2.5	0.5
28	Meering	Victoria	23.0	54.8	77.8	0.8	1.8	2.6	0.4
29	Silverstar	Victoria	11.2	31.6	42.8	0.4	1.2	1.7	0.4
30	Annuello	Victoria	17.0	38.9	55.9	0.7	1.7	2.4	0.4
31	Spear	South Australia	27.7	48.4	76.1	1.3	2.2	3.5	0.6
32	Frame	South Australia	27.3	65.4	92.7	1.3	3.1	4.4	0.4
33	Halberd	South Australia	37.1	72.1	109.2	1.6	3.2	4.8	0.5
34	Krichauff	South Australia	20.2	45.1	65.3	0.9	1.9	2.8	0.4
35	Kukri	South Australia	28.9	54.0	82.9	1.5	2.8	4.3	0.5

Supplemental 4.1 Germplasm origin and ACG traits (continued)

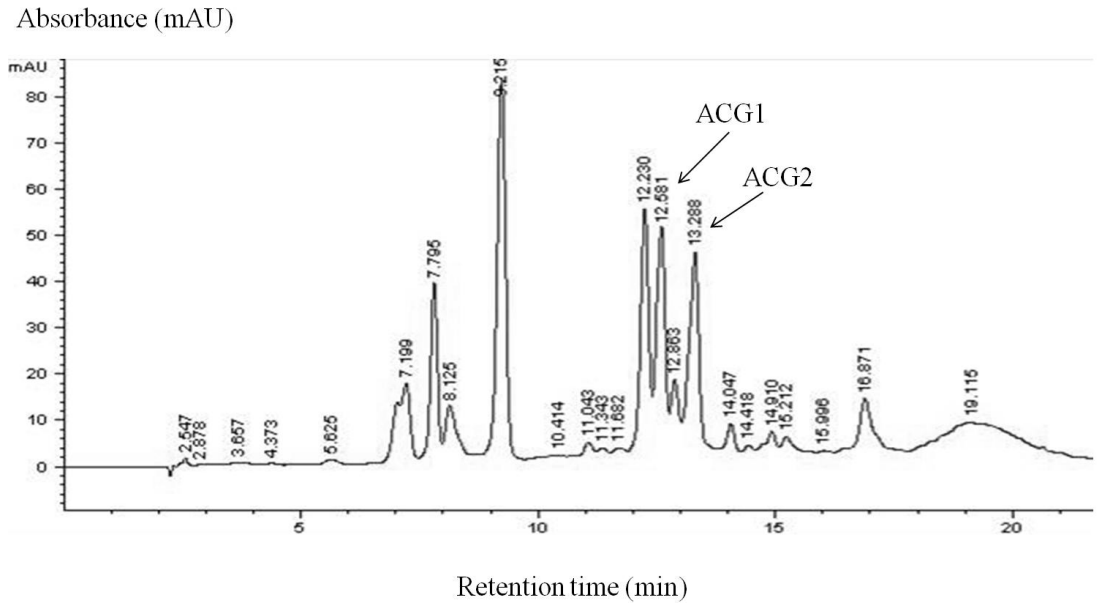
No	Cultivars	Origin	ACG1 (µg/g	ACG2 (µg/g	Total ACG (µg/g	ACG1 (µg/grain)	ACG2 (µg/grain)	Total ACG (µg/grain)	ACG1/ACG2 Ratio
36	Angus	South Australia	35.5	63.6	99.2	1.5	2.7	4.3	0.6
37	Aroona	South Australia	19.9	42.9	62.8	0.9	1.9	2.8	0.5
38	GBA Ruby	Western Australia	21.3	60.0	81.3	1.1	3.1	4.2	0.4
39	Cascades	Western Australia	18.9	42.8	61.8	0.9	2.0	2.9	0.4
40	Carnamah	Western Australia	19.7	43.4	63.1	1.0	2.2	3.2	0.4
41	Reeves	Western Australia	10.8	32.7	43.4	0.5	1.6	2.1	0.3
42	Westonia	Western Australia	18.4	43.8	62.2	0.8	1.8	2.6	0.4
43	Cranbrook	Western Australia	31.2	48.2	79.4	1.6	2.5	4.2	0.6
44	Canna	Western Australia	31.8	61.4	93.2	1.6	3.1	4.7	0.5
	Miscellaneous origin								
45	Seri	Mexico	21.1	52.0	73.1	1.1	2.7	3.7	0.4
46	Ciano	Mexico	24.0	35.5	59.5	1.3	1.9	3.1	0.7
47	AUS1408	South Africa	55.3	88.4	143.7	2.1	3.4	5.5	0.6
48	Indis	South Africa	26.0	44.1	70.1	0.8	1.4	2.3	0.6
49	SW95-50213	China	14.7	29.7	44.3	0.6	1.3	1.9	0.5
50	ChuanYu12	China	37.2	88.3	125.5	1.6	3.8	5.5	0.4
51	ChuanMai18	China	28.8	49.7	78.5	1.2	2.1	3.3	0.6
52	Jing Hong	China	30.6	74.0	104.6	1.9	4.5	6.4	0.4
53	Haruhikari	Japan	23.6	47.2	70.8	1.0	2.0	2.9	0.5
54	Chinese spring	China	52.4	80.7	133.2	2.7	4.1	6.7	0.7
55	SUN325B	Leslie Research Centre, Queensland	26.4	57.4	83.9	1.3	2.7	4.0	0.5
56	QT7475	Leslie Research Centre, Queensland	23.1	49.0	72.0	1.2	2.5	3.7	0.5
57	SW95-50213/Cunningham.763	University of Adelaide	35.7	66.9	102.6	1.2	2.2	3.3	0.5
58	SW95-50213/Cunningham.799	University of Adelaide	27.4	70.8	98.2	0.8	2.1	3.0	0.4
59	AUS 29565	Mexico	18.7	32.3	51.0	1.0	1.8	2.8	0.6
60	AUS 29604	Mexico	39.2	71.8	111.0	2.0	3.6	5.6	0.5
61	AUS 30330	Mexico	40.8	67.7	108.4	2.2	3.7	6.0	0.6
62	AUS 29572	Mexico	22.7	40.5	63.3	1.2	2.1	3.2	0.6
63	DM03.122/WI21121.1442	University of Adelaide	23.1	42.8	65.9	1.0	1.8	2.8	0.5
64	DM03.122/WI21121.1151	University of Adelaide	20.8	35.7	56.6	1.1	1.8	2.9	0.6
65	DM5686*B12	University of Adelaide	33.7	28.6	62.2	1.2	1.1	2.3	1.2
66	DM03.24.b.241	University of Adelaide	32.7	44.3	77.0	1.4	1.9	3.2	0.7
67	DM03.24.b.281	University of Adelaide	30.2	58.5	88.6	1.3	2.5	3.8	0.5
68	DM03.24.b.248	University of Adelaide	31.1	86.7	117.8	1.2	3.3	4.4	0.4
69	sunco/Indis.82	University of Adelaide	34.4	47.1	81.5	1.3	1.7	3.0	0.7
70	AUS1490	University of Adelaide	61.6	104.98	166.6	2.9	4.9	7.8	0.6

Supplemental 4.1 Germplasm origin and ACG traits (continued)

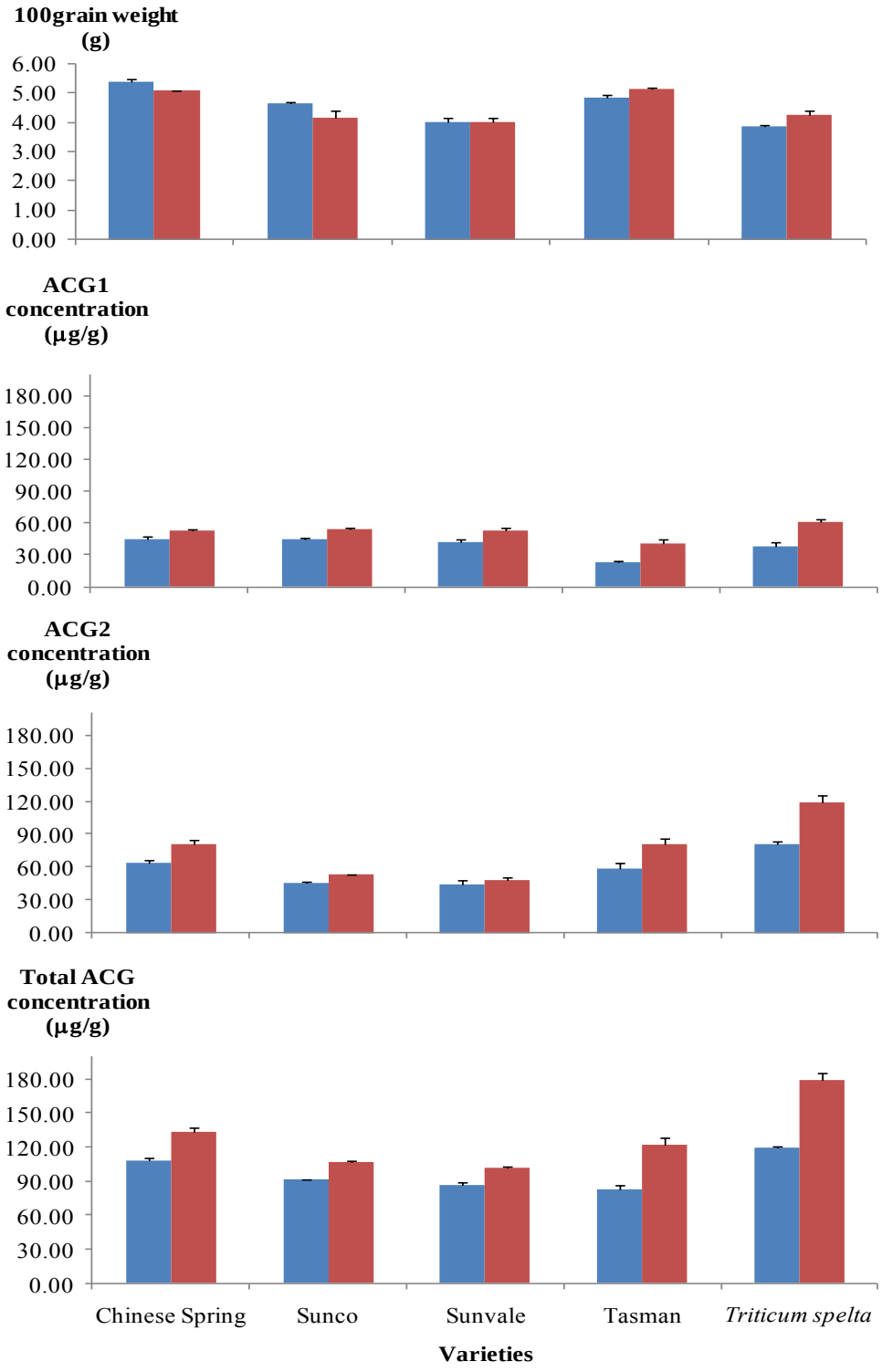
No	Cultivars	Origin	ACG1 (μg/g sample)	ACG2 (μg/g sample)	Total ACG (μg/g sample)	ACG1 (μg/grain)	ACG2 (μg/grain)	Total ACG (μg/grain)	ACG1/ACG2 Ratio
	Related species								
71	*T. monococcum* AUS90416	*Australian Winter Cereals Collection, Tamworth, NSW*	4.2	22.4	26.5	0.07	0.4	0.5	0.2
72	*T. monococcum* AUS90417	*Australian Winter Cereals Collection, Tamworth, NSW*	1.7	20.6	22.3	0.03	0.3	0.4	0.1
73	*T. monococcum* AUS19844	*Australian Winter Cereals Collection, Tamworth, NSW*	1.2	15.7	16.9	0.02	0.3	0.3	0.1
74	*T. monococcum* AUS90359	*Australian Winter Cereals Collection, Tamworth, NSW*	1.9	21.4	23.3	0.05	0.6	0.6	0.1
75	*T. monococcum* AUS90357	*Australian Winter Cereals Collection, Tamworth, NSW*	3.5	12.3	15.8	0.07	0.3	0.3	0.3
76	*Aegilops tauschii* AUS24062	*Australian Winter Cereals Collection, Tamworth, NSW*	10.6	14.4	25.0	0.08	0.1	0.2	0.7
77	*Aegilops tauschii* AUS24091	*Australian Winter Cereals Collection, Tamworth, NSW*	9.2	11.1	20.4	0.07	0.1	0.2	0.8
78	*Aegilops tauschii* AUS23990	*Australian Winter Cereals Collection, Tamworth, NSW*	17.9	20.8	38.7	0.26	0.3	0.6	0.9
79	*Aegilops tauschii* AUS23980	*Australian Winter Cereals Collection, Tamworth, NSW*	13.8	17.7	31.6	0.17	0.2	0.4	0.8
80	*Aegilops tauschii* AUS24048	*Australian Winter Cereals Collection, Tamworth, NSW*	15.5	22.0	37.5	0.19	0.3	0.5	0.7
81	*Aegilops tauschii* AUS22445	*Australian Winter Cereals Collection, Tamworth, NSW*	24.1	38.6	62.7	1.17	1.9	3.0	0.6
82	*T. uratu* AUS27031	*Australian Winter Cereals Collection, Tamworth, NSW*	4.7	40.5	45.1	0.10	0.9	0.9	0.1
83	*T. uratu* AUS26932	*Australian Winter Cereals Collection, Tamworth, NSW*	5.4	34.3	39.8	0.13	0.8	0.9	0.2
84	*T. uratu* AUS26939	*Australian Winter Cereals Collection, Tamworth, NSW*	4.5	35.4	40.0	0.10	0.8	0.9	0.1
85	*T. uratu* AUS26947	*Australian Winter Cereals Collection, Tamworth, NSW*	1.9	15.3	17.2	0.04	0.3	0.4	0.1
86	*T. uratu* AUS26978	*Australian Winter Cereals Collection, Tamworth, NSW*	6.1	43.9	50.0	0.13	0.9	1.0	0.1
87	*Aegilops speltoides* AUS18813	*Australian Winter Cereals Collection, Tamworth, NSW*	29.6	39.6	69.2	0.14	0.2	0.3	0.7
88	*Aegilops speltoides* AUS18995	*Australian Winter Cereals Collection, Tamworth, NSW*	23.4	52.6	77.9	0.16	0.3	0.5	0.5
89	*Aegilops speltoides* AUS20346	*Australian Winter Cereals Collection, Tamworth, NSW*	34.8	60.4	95.2	0.13	0.2	0.4	0.6
90	*Aegilops speltoides* AUS21637	*Australian Winter Cereals Collection, Tamworth, NSW*	18.3	31.4	49.7	0.24	0.4	0.7	0.6
91	*Aegilops speltoides* AUS21640	*Australian Winter Cereals Collection, Tamworth, NSW*	14.5	28.2	42.7	0.07	0.1	0.2	0.5
92	*Triticum diccocum* AUS17641	*Australian Winter Cereals Collection, Tamworth, NSW*	30.3	78.0	108.3	1.19	3.1	4.3	0.4
93	*Triticum diccocum* var rufum AUS21758	*Australian Winter Cereals Collection, Tamworth, NSW*	31.0	44.3	75.3	1.53	2.2	3.7	0.7
94	*Triticum diccocum* var farrum AUS10559	*Australian Winter Cereals Collection, Tamworth, NSW*	33.1	72.2	105.3	1.08	2.4	3.5	0.5
95	*Triticum dicoccoides* AUS17967	*Australian Winter Cereals Collection, Tamworth, NSW*	44.6	67.0	111.5	2.38	3.6	6.0	0.7
96	*Triticum carthlicum* AUS17644	*Australian Winter Cereals Collection, Tamworth, NSW*	26.7	100.1	126.8	1.33	5.0	6.3	0.3
97	*Triticum polonicum* AUS17970	*Australian Winter Cereals Collection, Tamworth, NSW*	27.0	81.5	108.5	1.31	3.9	5.3	0.3
98	*Triticum polonicum* AUS17645	*Australian Winter Cereals Collection, Tamworth, NSW*	25.8	59.8	85.7	0.99	2.3	3.3	0.4
99	*Triticum turgidum* AUS17969	*Australian Winter Cereals Collection, Tamworth, NSW*	37.2	67.5	104.7	1.61	2.9	4.5	0.6
100	*Triticum durum* var *bellaroi*	*NSW*	17.5	54.1	71.6	1.09	3.4	4.5	0.3
101	*Triticum durum* var *wollaroi*	*NSW*	20.1	33.8	53.9	1.27	2.1	3.4	0.6
102	*Triticum durum* var *kalka*	*South Australia*	29.8	49.1	78.9	1.55	2.5	4.1	0.6
103	*Triticum durum* var *yallaroi*	*NSW*	33.5	65.0	98.4	1.51	2.9	4.4	0.5
104	*Triticum durum* var *kamilaroi*	*NSW*	28.7	56.5	85.2	1.41	2.8	4.2	0.5
105	*Triticum spelta*	*Australian Winter Cereals Collection, Tamworth, NSW*	60.6	118.8	179.4	2.56	5.0	7.6	0.5

* AUS: accession number in Australian winter cereal collection (AWCC)

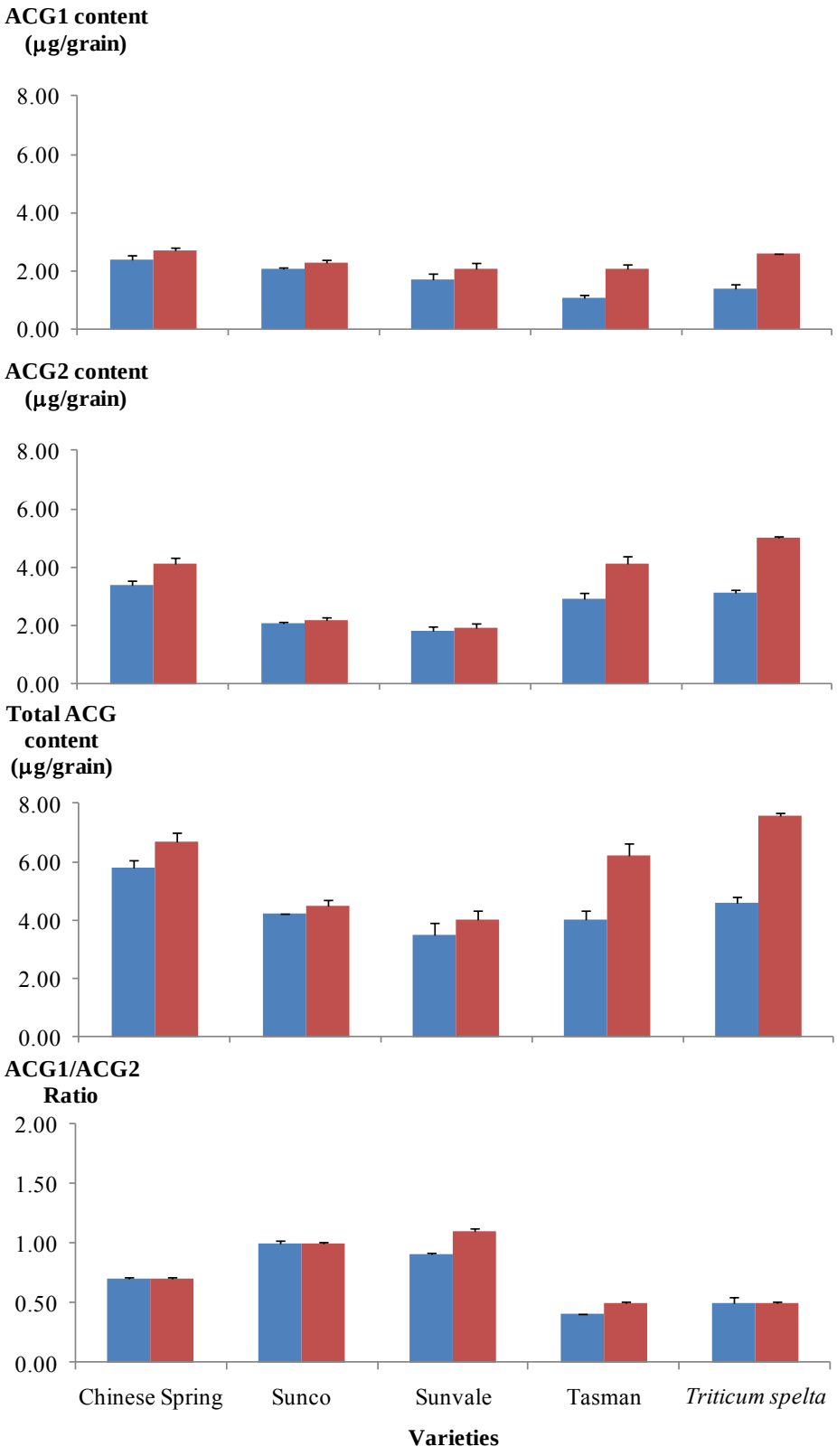
Supplementary material 4.2 Chromatogram obtained for neutral hydroxylamine extract of *T. aestivum* cv Sunco. The peaks corresponding to the two types of the apigenin *C*-di-glycosides (ACG1 and ACG2) are indicated by arrows.



Supplemental material 4.3 100 grain weight, ACG consentration, ACG content and ratio ofsome variaties that were grown both in the field and in the glass house. Blue box: in the field; red box: inthe glass house, bars represnt SE of means.



Supplemental material 4.3 (continued)



Supplemental material 4.4 Means (± SE) of total ACG content (µg/grain) of nullisomic (N)-tetrasomic (T) lines Chinese Spring (A) and Chinese Spring/Triticum spelta substitution lines (B). a-n: a-i: indicate significant differences ($p<0.01$) in total ACG content based on Tukey test of multiple comparison at 5% level.

A

Nullisomic-tetrasomic lines of Chines Spring	Total ACG content (µg/grain)	
N 1A T 1B	5.75 ± 0.80	jklm
N 1A T 1D	5.45 ± 0.28	ijklm
N 1B T 1A	3.74 ± 0.06	abcdefghi
N 1B T 1D	3.26 ± 0.04	abcdefg
N 1D T 1A	4.01 ± 0.11	abcdefghijk
N 1D T 1B	4.74 ± 0.23	fghijklm
N 2A T 2B	5.98 ± 0.27	lmn
N 2A T 2D	3.76 ± 0.18	abcdefghi
N 2B T 2A	2.99 ± 0.14	abcdef
N 2B T 2D	2.53 ± 0.09	abcd
N 2D T 2A	4.44 ± 0.18	defghijklm
N 2D T 2B	4.22 ± 0.24	bcdefghijkl
N 3A T 3B	3.72 ± 0.11	abcdefghi
N 3A T 3D	5.10 ± 0.39	ghijklm
N 3B T 3A	5.50 ± 0.19	ijklm
N 3B T 3D	4.26 ± 0.21	cdefghijkl
N 3D T 3A	4.71 ± 0.24	fghijklm
N 3D T 3B	4.23 ± 0.13	cdefghijkl
N 4A T 4B	3.43 ± 0.76	abcdefgh
N 4A T 4D	4.35 ± 0.46	cdefghijklm
N 4B T 4A	4.55 ± 0.26	efghijklm
N 4B T 4D	5.91 ± 0.67	klmn
N 4D T 4A	2.14 ± 0.19	a
N 4D T 4B	4.23 ± 0.07	cdefghijkl
N 5A T 5B	3.46 ± 0.29	abcdefgh
N 5A T 5D	3.36 ± 0.22	abcdefgh
N 5B T 5A	4.41 ± 0.52	cdefghijklm
N 5B T 5D	4.96 ± 0.40	ghijklm
N 5D T 5A	2.28 ± 0.10	ab
N 5D T 5B	3.42 ± 0.19	abcdefgh
N 6A T 6B	5.28 ± 0.20	hijklm
N 6A T 6D	5.08 ± 0.69	ghijklm
N 6B T 6A	2.69 ± 0.31	abcde
N 6B T 6D	5.23 ± 0.56	hijklm
N 6D T 6A	2.50 ± 0.24	abc
N 6D T 6B	3.93 ± 0.21	abcdefghij
N 7A T 7B	3.16 ± 0.05	abcdefg
N 7A T 7D	7.73 ± 0.30	n
N 7B T 7A	6.21 ± 0.45	mn
N 7B T 7D	6.13 ± 0.39	lmn
N 7D T 7A	5.41 ± 0.13	ijklm
N 7D T 7B	2.59 ± 0.21	abcd
Chinese spring	5.82 ± 0.28	jklmn

B

Substitution lines	Total ACG content (µg/grain)	
1A	7.79 ± 0.44	bcde
1B	5.97 ± 0.25	ab
1D	8.47 ± 0.54	de
2A	6.40 ± 0.35	abcd
2B	8.23 ± 0.81	bcde
2D	7.81 ± 0.16	bcde
3A	6.68 ± 0.16	abcd
3B	6.84 ± 0.24	abcde
3D	7.95 ± 0.50	bcde
4A	8.40 ± 0.42	cde
4B	7.56 ± 0.40	bcde
4D	6.66 ± 0.06	abcd
5A	6.12 ± 0.26	abc
5B	6.29 ± 0.21	abcd
5D	6.78 ± 0.68	abcd
6A	8.16 ± 0.26	bcde
6B	6.62 ± 0.16	abcd
6D	5.22 ± 0.26	a
7A	6.61 ± 0.27	abcd
7B	9.10 ± 0.98	e
7D	6.20 ± 0.42	abcd
Chinese spring	6.74 ± 0.32	abcd
Triticum spelta	7.59 ± 0.08	bcde

Supplemental material 4.5 Pedigree of Sunco and its relatives as drawn from (Martynov & Dobrotvorskaya 2006).

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Supplemental material 4.6 Pedigree of Tasman and its relatives as drawn from (Martynov & Dobrotvorskaya 2006).

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Supplemental material 5.1 Proportion and ratio of ACG1 and ACG2 in grain and Quadrumat Junior mill flour.

Cultivars	Proportion in grain (%)		Ratio in grain	Proportion in flour (%)		Ratio in flour
	ACG1	ACG2		ACG1	ACG2	
Ventura	27.2 ± 0.3	72.8 ± 0.3	0.4 ± 0.005	42.7 ± 0.3	57.3 ± 0.3	0.7 ± 0.01
Sunvale	49.0 ± 0.3	51.0 ± 0.3	1.0 ± 0.01	56.1 ± 0.5	43.9 ± 0.5	1.3 ± 0.03
Sunco	49.7 ± 0.4	50.3 ± 0.4	1.0 ± 0.02	54.7 ± 1.6	45.3 ± 1.6	1.2 ± 0.1
Diamondbird	25.3 ± 0.5	74.7 ± 0.5	0.3 ± 0.01	46.9 ± 2.3	53.1 ± 2.3	0.9 ± 0.1
Sunlin	35.5 ± 2.3	64.5 ± 2.3	0.6 ± 0.1	43.7 ± 0.6	56.3 ± 0.6	0.8 ± 0.02
Cunningham	32.0 ± 0.5	68.0 ± 0.5	0.5 ± 0.01	44.4 ± 1.9	55.6 ± 1.9	0.8 ± 0.1
EGA Gregory	29.5 ± 2.3	70.5 ± 2.3	0.4 ± 0.05	50.7 ± 2.2	49.3 ± 2.2	1.0 ± 0.1
Hartog	29.9 ± 0.7	70.1 ± 0.7	0.4 ± 0.01	37.4 ± 0.5	62.6 ± 0.5	0.6 ± 0.01
Janz	41.9 ± 1.2	58.1 ± 1.2	0.7 ± 0.04	52.2 ± 4.1	47.8 ± 4.1	1.1 ± 0.2
EGA Hume	31.7 ± 0.4	68.3 ± 0.4	0.5 ± 0.01	38.4 ± 1.1	61.6 ± 1.1	0.6 ± 0.03
Kennedy	24.9 ± 0.1	75.1 ± 0.1	0.3 ± 0.002	37.9 ± 0.3	62.1 ± 0.3	0.6 ± 0.01
Lang	32.2 ± 0.5	67.8 ± 0.5	0.5 ± 0.01	45.9 ± 0.1	54.1 ± 0.1	0.8 ± 0.003
Batavia	29.4 ± 0.6	70.6 ± 0.6	0.4 ± 0.01	44.0 ± 2.6	56.0 ± 2.6	0.8 ± 0.1
Baxter	43.8 ± 0.1	56.2 ± 0.1	0.8 ± 0.004	57.0 ± 2.5	43.0 ± 2.5	1.3 ± 0.1
Spear	36.4 ± 0.3	63.6 ± 0.3	0.6 ± 0.01	50.6 ± 0.6	49.4 ± 0.6	1.0 ± 0.02
Frame	29.4 ± 0.1	70.6 ± 0.1	0.4 ± 0.002	40.9 ± 1.1	59.1 ± 1.1	0.7 ± 0.03
Halberd	34.0 ± 0.5	66.0 ± 0.5	0.5 ± 0.01	45.0 ± 0.6	55.0 ± 0.6	0.8 ± 0.02
Krichauff	31.0 ± 0.3	69.0 ± 0.3	0.4 ± 0.01	39.1 ± 1.1	60.9 ± 1.1	0.6 ± 0.03
Kukri	34.8 ± 0.1	65.2 ± 0.1	0.5 ± 0.003	45.7 ± 2.8	54.3 ± 2.8	0.9 ± 0.1
Chara	32.1 ± 0.7	67.9 ± 0.7	0.5 ± 0.01	36.3 ± 1.8	63.7 ± 1.8	0.6 ± 0.04
Meering	29.6 ± 0.6	70.4 ± 0.6	0.4 ± 0.01	38.5 ± 1.0	61.5 ± 1.0	0.6 ± 0.03
Silverstar	26.3 ± 1.6	73.7 ± 1.6	0.4 ± 0.03	41.4 ± 1.8	58.6 ± 1.8	0.7 ± 0.1
Annuello	30.3 ± 0.6	69.7 ± 0.6	0.4 ± 0.01	45.1 ± 1.1	54.9 ± 1.1	0.8 ± 0.04
GBA Ruby	26.2 ± 0.0	73.8 ± 0.0	0.4 ± 0.001	39.4 ± 1.3	60.6 ± 1.3	0.7 ± 0.03
Cascades	30.6 ± 0.3	69.4 ± 0.3	0.4 ± 0.01	42.7 ± 0.2	57.3 ± 0.2	0.7 ± 0.00
Carnamah	31.0 ± 0.7	69.0 ± 0.7	0.4 ± 0.01	45.0 ± 1.6	55.0 ± 1.6	0.8 ± 0.1
Reeves	24.8 ± 0.7	75.2 ± 0.7	0.3 ± 0.01	36.2 ± 1.9	63.8 ± 1.9	0.6 ± 0.05
Westonia	29.6 ± 0.2	70.4 0.2	0.4 0.005	49.6 ± 0.5	50.4 ± 0.5	1.0 ± 0.02
Max	49.7	75.2	1.0	57.0	63.8	1.3
Min	24.8	50.3	0.3	36.2	43.0	0.6
Means	32.4 ± 3.4	67.6 ± 3.4	0.5 ± 0.1	44.6 ± 3.8	55.4 ± 3.8	0.8 ± 0.1

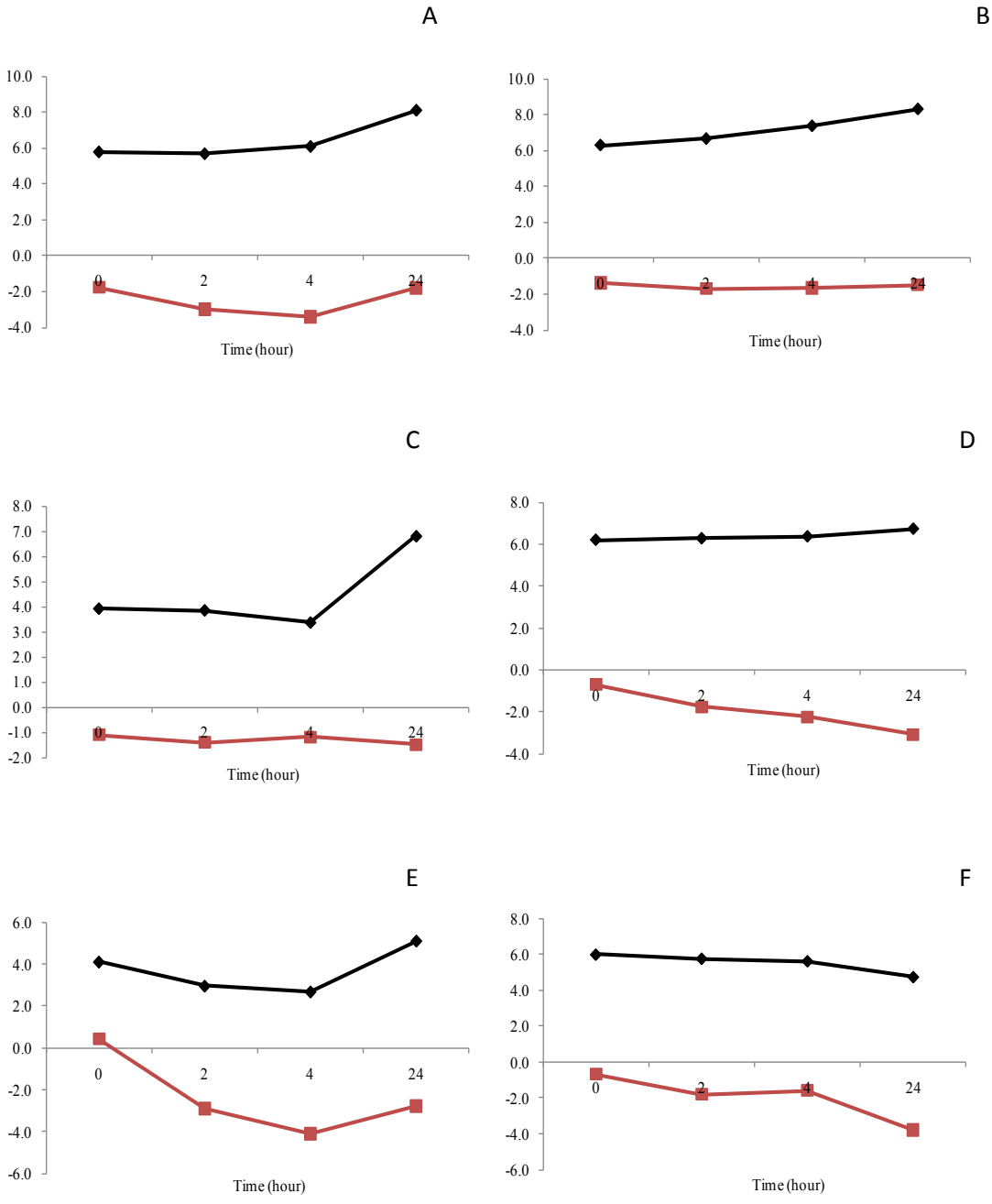
Supplemental material 5.2 Proportion (%) of Lutein in grain and Quadrumat Junior mill flour.

Varieties	Proportion in the grain (%)				Proportion in the flour (%)			
	Lutein	Monoester	Diester	Lutein-ester	Lutein	Monoester	Diester	Lutein-ester
Ventura	33.2 ± 0.01	25.3 ± 0.40	41.5 ± 0.40	66.8 ± 0.01	23.1 ± 0.07	28.9 ± 0.10	48.0 ± 0.1	76.9 ± 0.1
Sunvale	35.9 ± 0.82	37.8 ± 1.32	26.2 ± 0.51	64.1 ± 0.82	24.9 ± 0.08	34.8 ± 0.20	40.3 ± 0.3	75.1 ± 0.1
Sunco	33.4 ± 0.18	35.0 ± 0.18	31.7 ± 0.21	66.6 ± 0.18	25.6 ± 0.13	31.9 ± 0.29	42.5 ± 0.3	74.4 ± 0.1
Diamondbird	34.7 ± 0.12	29.2 ± 0.26	36.1 ± 0.24	65.3 ± 0.12	24.0 ± 0.08	30.8 ± 0.03	45.2 ± 0.1	76.0 ± 0.1
Sunlin	30.4 ± 0.08	32.3 ± 0.12	37.3 ± 0.14	69.6 ± 0.08	21.5 ± 0.06	36.6 ± 0.01	41.9 ± 0.1	78.5 ± 0.1
Cunningham	33.4 ± 0.27	35.5 ± 0.20	31.1 ± 0.22	66.6 ± 0.27	27.0 ± 0.24	36.2 ± 0.06	36.8 ± 0.3	73.0 ± 0.2
EGA Gregory	32.2 ± 0.21	31.5 ± 0.13	36.4 ± 0.31	67.8 ± 0.21	25.0 ± 0.08	34.4 ± 0.20	40.6 ± 0.3	75.0 ± 0.1
Hartog	38.3 ± 0.39	42.4 ± 0.31	19.3 ± 0.11	61.7 ± 0.39	27.8 ± 0.08	33.5 ± 0.04	38.7 ± 0.1	72.2 ± 0.1
Janz	32.8 ± 0.01	33.9 ± 0.14	33.3 ± 0.13	67.2 ± 0.01	22.7 ± 0.18	36.1 ± 0.34	41.1 ± 0.4	77.3 ± 0.2
EGA Hume	38.0 ± 0.23	31.6 ± 0.37	30.4 ± 0.14	62.0 ± 0.23	25.2 ± 0.31	31.3 ± 0.19	43.5 ± 0.4	74.8 ± 0.3
Kennedy	30.8 ± 0.12	28.8 ± 0.25	40.4 ± 0.14	69.2 ± 0.12	19.1 ± 0.04	27.2 ± 0.26	53.8 ± 0.2	80.9 ± 0.0
Lang	33.1 ± 0.23	35.0 ± 0.31	31.9 ± 0.13	66.9 ± 0.23	23.1 ± 0.11	35.1 ± 0.33	41.8 ± 0.3	76.9 ± 0.1
Batavia	32.6 ± 0.13	34.0 ± 0.17	33.3 ± 0.09	67.4 ± 0.13	24.2 ± 0.07	36.6 ± 0.22	39.2 ± 0.2	75.8 ± 0.1
Baxter	36.5 ± 0.15	32.8 ± 0.20	30.7 ± 0.22	63.5 ± 0.15	25.2 ± 0.25	31.3 ± 0.12	43.5 ± 0.3	74.8 ± 0.2
Spear	37.4 ± 0.02	34.8 ± 0.16	27.8 ± 0.16	62.6 ± 0.02	31.0 ± 0.11	34.3 ± 0.25	34.6 ± 0.2	69.0 ± 0.1
Frame	40.6 ± 0.34	44.0 ± 0.14	15.4 ± 0.39	59.4 ± 0.34	32.2 ± 0.06	32.3 ± 0.02	35.5 ± 0.1	67.8 ± 0.1
Halberd	38.1 ± 0.30	39.7 ± 0.44	22.3 ± 0.73	61.9 ± 0.30	30.2 ± 0.08	35.2 ± 0.15	34.6 ± 0.1	69.8 ± 0.1
Krichauff	30.9 ± 0.07	35.2 ± 0.01	33.9 ± 0.06	69.1 ± 0.07	27.8 ± 0.11	37.3 ± 0.16	35.0 ± 0.3	72.2 ± 0.1
Kukri	38.2 ± 0.37	34.4 ± 0.65	27.3 ± 0.31	61.8 ± 0.37	30.5 ± 0.13	37.8 ± 0.03	31.8 ± 0.1	69.5 ± 0.1
Chara	32.7 ± 0.36	30.2 ± 0.45	37.1 ± 0.11	67.3 ± 0.36	24.3 ± 0.19	35.4 ± 0.13	40.3 ± 0.1	75.7 ± 0.2
Meering	36.3 ± 0.39	28.8 ± 0.10	34.9 ± 0.31	63.7 ± 0.39	26.0 ± 0.07	34.5 ± 0.06	39.5 ± 0.1	74.0 ± 0.1
Silverstar	39.3 ± 0.11	34.5 ± 0.10	26.2 ± 0.12	60.7 ± 0.11	28.8 ± 0.08	35.3 ± 0.29	35.9 ± 0.3	71.2 ± 0.1
Annuello	31.4 ± 0.15	39.5 ± 0.46	29.1 ± 0.33	68.6 ± 0.15	27.0 ± 0.07	33.7 ± 0.12	39.3 ± 0.1	73.0 ± 0.1
GBA Ruby	35.0 ± 0.21	32.1 ± 0.28	32.8 ± 0.19	65.0 ± 0.21	18.9 ± 0.08	33.5 ± 0.19	47.7 ± 0.2	81.1 ± 0.1
Cascades	33.6 ± 0.19	35.2 ± 0.20	31.2 ± 0.07	66.4 ± 0.19	26.7 ± 0.05	37.2 ± 0.10	36.1 ± 0.1	73.3 ± 0.1
Carnamah	26.4 ± 0.37	34.2 ± 0.05	39.5 ± 0.36	73.6 ± 0.37	21.0 ± 0.10	31.6 ± 0.09	47.4 ± 0.1	79.0 ± 0.1
Reeves	30.5 ± 0.36	31.5 ± 0.94	38.0 ± 0.59	69.5 ± 0.36	17.9 ± 0.07	31.3 ± 0.13	50.8 ± 0.2	82.1 ± 0.1
Westonia	30.1 ± 0.03	32.6 ± 0.04	37.4 ± 0.06	69.9 ± 0.03	23.8 ± 0.13	33.4 ± 0.01	42.8 ± 0.1	76.2 ± 0.1
Max	40.6	44.0	41.5	73.6	32.2	37.8	53.8	82.1
Min	26.4	25.3	15.4	59.4	17.9	27.2	31.8	67.8
Means	34.1 ± 0.63	34.0 ± 0.77	31.9 ± 1.17	65.9 ± 0.63	25.2 ± 0.68	33.8 ± 0.49	41.0 ± 1.0	74.8 ± 0.7

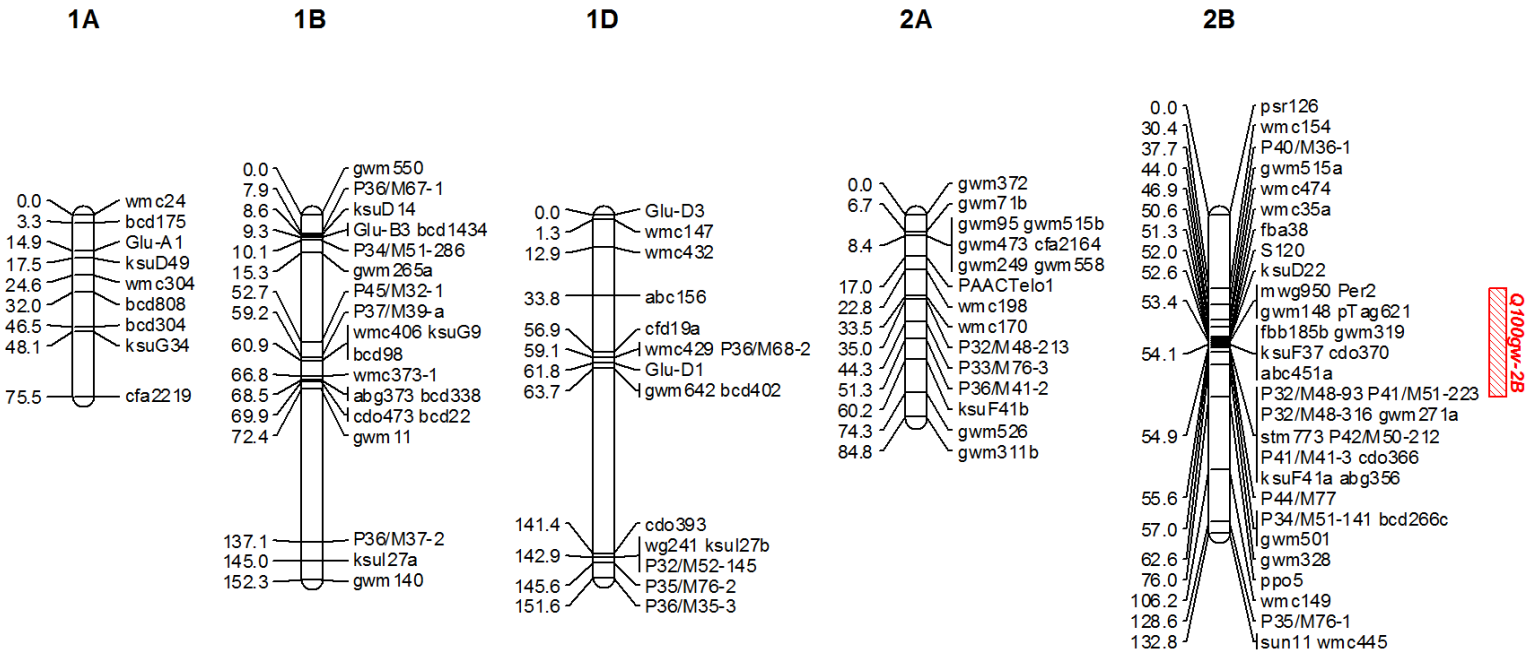
Supplemental material 5.3 ACG content and recovery in Bühler Mill flour fractions.

ACG Traits	Sample	Concentration (μg/g) in samples				Total ACG concentration in flour fractions (μg/g)	Total ACG concentration in grain (μg/g)	The amount of ACG (μg) in milling fractions				Total amount of ACG in flour fractions (μg)	Total amount ACG in all milling fraction (μg)	Recovery in flour fractions (%)	Recovery in Bran and pollard fractions (%)
		Break flour (μg/g)	Reduction flour (μg/g)	Bran (μg/g)	Pollard (μg/g)			Break flour (μg)	Reduction flour (μg)	Bran (μg)	Pollard (μg)				
ACG1	Krichauff	0.7	2.6	88.8	98.3	3.3	190.4	0.3	6.4	85.6	77.5	6.7	169.8	4.0	96.0
	Silver star	1.8	2.9	51.2	79.5	4.7	135.4	1.4	9.3	54.5	46.5	10.7	111.7	9.6	90.4
	Halberd	1.8	6.7	149.3	136.7	8.5	294.5	0.8	13.6	107.4	40.8	14.4	162.6	8.9	91.1
	Sunco	2.1	6.2	94.2	73.2	8.3	175.7	1.4	20.4	89.9	37.0	21.8	148.8	14.7	85.3
	Ruby	2.2	5.4	105.2	114.9	7.6	227.7	1.3	16.4	90.4	57.2	17.7	165.3	10.7	89.3
	Hartog	2.7	2.8	61.8	86.2	5.5	153.5	1.5	6.7	45.2	35.7	8.2	89.0	9.2	90.8
	Reeves	1.9	5.6	51.1	84.3	7.5	142.9	0.7	12.0	37.7	48.1	12.7	98.5	12.9	87.1
	Aroona	2.5	10.0	47.7	111.6	12.4	171.8	1.9	31.5	44.2	48.0	33.4	125.6	26.6	73.4
	Sunvale	1.2	8.2	84.1	146.7	9.4	240.1	0.7	20.3	66.0	61.9	21.1	149.0	14.1	85.9
	Hume	0.8	2.0	101.9	99.9	2.8	204.6	0.5	5.3	100.8	52.8	5.8	159.5	3.7	96.3
	Max	2.7	10.0	149.3	146.7	12.4	294.5	1.9	31.5	107.4	77.5	33.4	169.8	26.6	96.3
	Min	0.7	2.0	47.7	73.2	2.8	135.4	0.3	5.3	37.7	35.7	5.8	89.0	3.7	73.4
	Rates	1.8	5.2	83.5	103.1	7.0	193.7	1.1	14.2	72.2	50.5	15.3	138.0	11.4	88.6
ACG2	Krichauff	1.0	4.7	128.2	183.3	5.8	317.2	0.5	11.5	123.6	144.5	12.0	280.2	4.3	95.7
	Silver star	3.4	5.2	113.2	165.6	8.7	287.5	2.7	16.8	120.5	96.8	19.4	236.7	8.2	91.8
	Halberd	2.1	9.7	222.5	201.1	11.7	435.3	0.9	19.6	160.0	60.0	20.5	240.5	8.5	91.5
	Sunco	1.7	6.2	70.9	69.4	7.9	148.2	1.1	20.6	67.7	35.1	21.7	124.5	17.5	82.5
	Ruby	6.7	10.8	270.6	265.4	17.5	553.5	4.0	33.1	232.6	132.1	37.1	401.7	9.2	90.8
	Hartog	4.7	5.8	148.6	196.4	10.5	355.6	2.7	13.7	108.7	81.2	16.4	206.3	8.0	92.0
	Reeves	3.6	11.7	106.9	173.6	15.3	295.9	1.3	25.2	78.9	99.1	26.4	204.5	12.9	87.1
	Aroona	3.8	17.3	98.0	199.7	21.1	318.8	2.9	54.7	90.8	85.9	57.6	234.3	24.6	75.4
	Sunvale	1.1	8.7	121.3	154.2	9.8	285.3	0.6	21.6	95.3	65.1	22.2	182.6	12.2	87.8
	Hume	1.3	2.8	145.1	165.9	4.1	315.2	0.9	7.6	143.5	87.8	8.5	239.8	3.5	96.5
	Max	6.7	17.3	270.6	265.4	21.1	553.5	4.0	54.7	232.6	144.5	57.6	401.7	24.6	96.5
	Min	1.0	2.8	70.9	69.4	4.1	148.2	0.5	7.6	67.7	35.1	8.5	124.5	3.5	75.4
	Rates	2.9	8.3	142.5	177.5	11.3	331.2	1.8	22.4	122.1	88.8	24.2	235.1	10.9	89.1
Total ACG content	Krichauff	1.7	7.4	217.0	281.5	9.1	507.6	0.9	17.9	209.2	222.1	18.7	450.0	4.2	95.8
	Silver star	5.2	8.2	164.4	245.2	13.4	422.9	4.1	26.1	175.0	143.2	30.2	348.4	8.7	91.3
	Halberd	3.8	16.3	371.8	337.7	20.2	729.7	1.7	33.2	267.4	100.8	34.9	403.1	8.7	91.3
	Sunco	3.7	12.4	165.1	142.6	16.2	323.9	2.6	41.0	157.6	72.1	43.6	273.3	15.9	84.1
	Ruby	8.9	16.2	375.8	380.3	25.1	781.2	5.4	49.4	323.0	189.2	54.8	567.0	9.7	90.3
	Hartog	7.4	8.6	210.4	282.7	16.1	509.2	4.2	20.4	153.8	116.9	24.6	295.3	8.3	91.7
	Reeves	5.6	17.3	158.1	257.9	22.8	438.8	2.0	37.2	116.7	147.2	39.1	303.0	12.9	87.1
	Aroona	6.2	27.3	145.7	311.3	33.5	490.5	4.8	86.2	135.0	133.9	91.0	359.9	25.3	74.7
	Sunvale	2.3	17.0	205.3	300.8	19.3	525.4	1.4	41.9	161.3	127.1	43.3	331.6	13.0	87.0
	Hume	2.1	4.8	247.0	265.8	6.9	519.7	1.5	12.9	244.3	140.7	14.3	399.3	3.6	96.4
	Max	8.9	27.3	375.8	380.3	33.5	781.2	5.4	86.2	323.0	222.1	91.0	567.0	25.3	96.4
	Min	1.7	4.8	145.7	142.6	6.9	323.9	0.9	12.9	116.7	72.1	14.3	273.3	3.6	74.7
	Rates	4.7	13.6	226.1	280.6	18.3	524.9	2.8	36.6	194.3	139.3	39.4	373.1	11.0	89.0

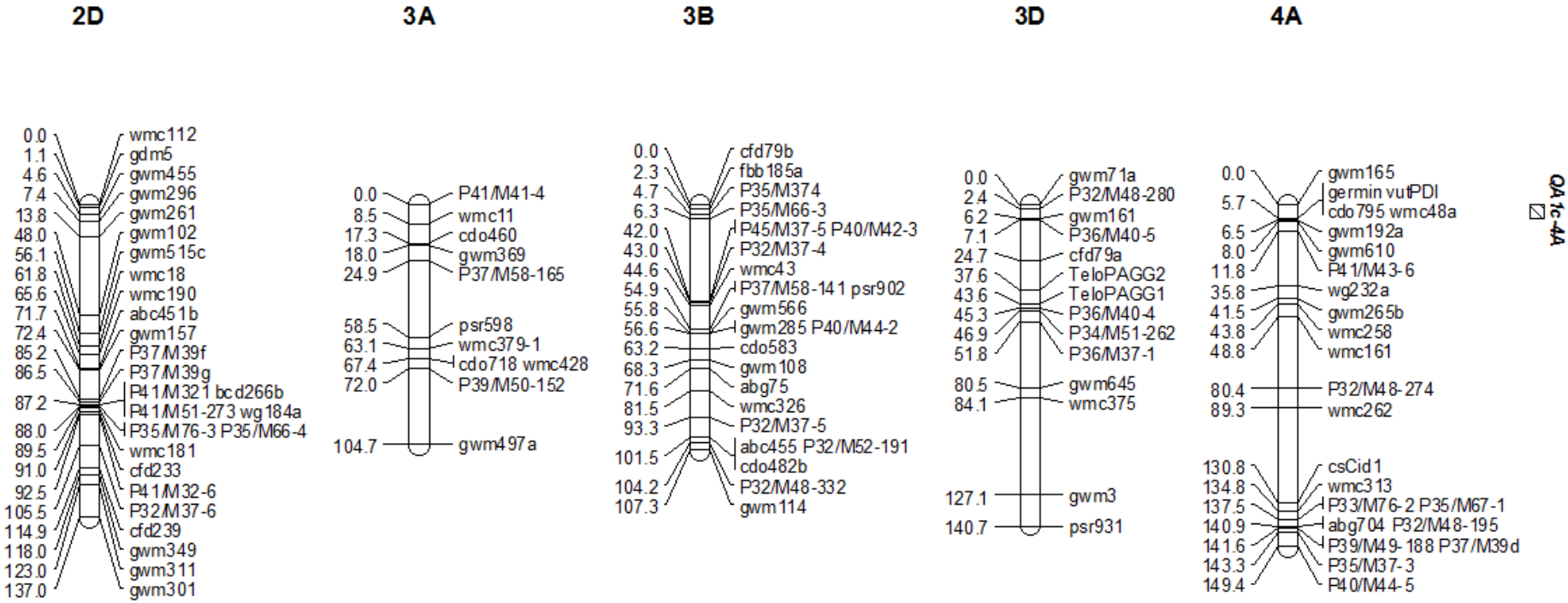
Supplemental material 5.4 Comparison of changes in the ΔL* (YAN-WSN, red lines and markers) and Δb* (YAN-WSN, black lines and markers) at 0, 2, 4 and 24 hours. A: Sunvale, B:Sunco, C: Krichauff, D: GBA Ruby, E: Carnamah, F:Reeves.



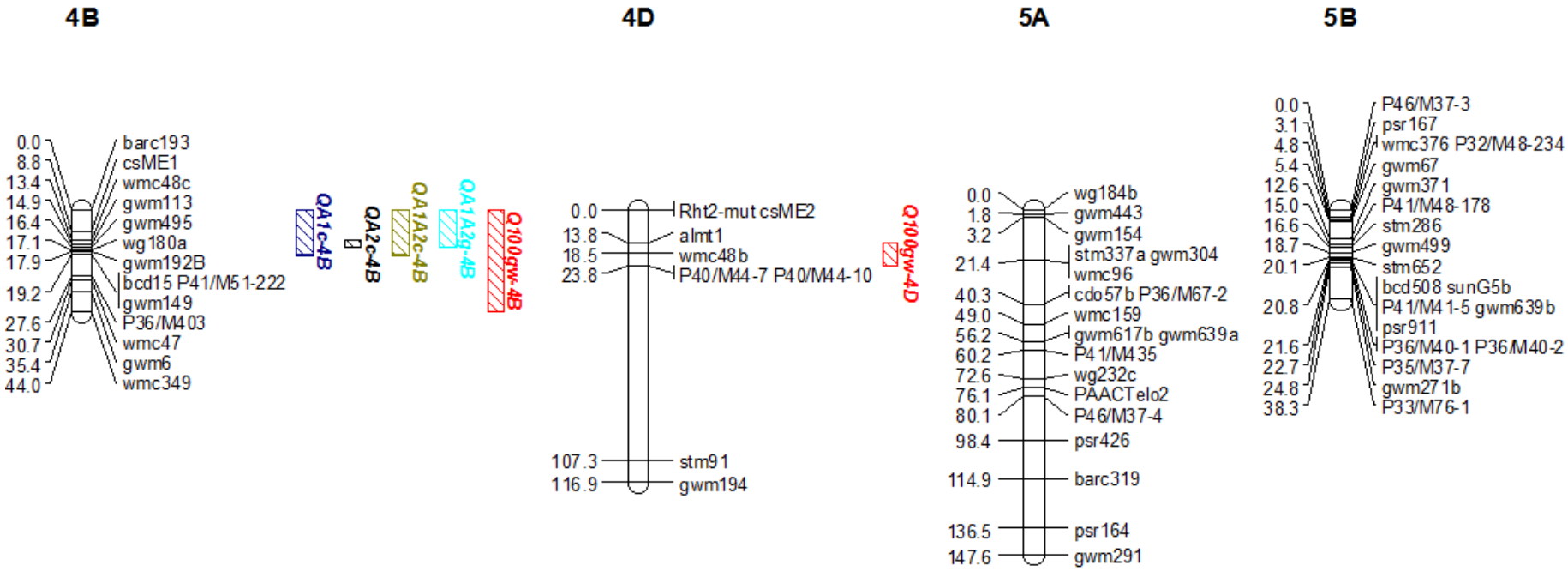
Supplemental material 6.1 Genetic map of Sunco/Tasman doubled haploid population re-constructed with 361 markers and 153 lines of the population. The bars next to the 2B, 4A, 4B, 4D and 7B maps show the QTL location: ACG1 concentration (µg/g) (dark blue), ACG2 contentration (µg/g) (black), ACG1 content (µg/grain) (green), ACG2 content (µg/grain) (pink), ACG1/ACG2 ratio (brown), 100 grain weight (red), total concentration (µg/g) (gold) and total content (µg/grain): bright blue.



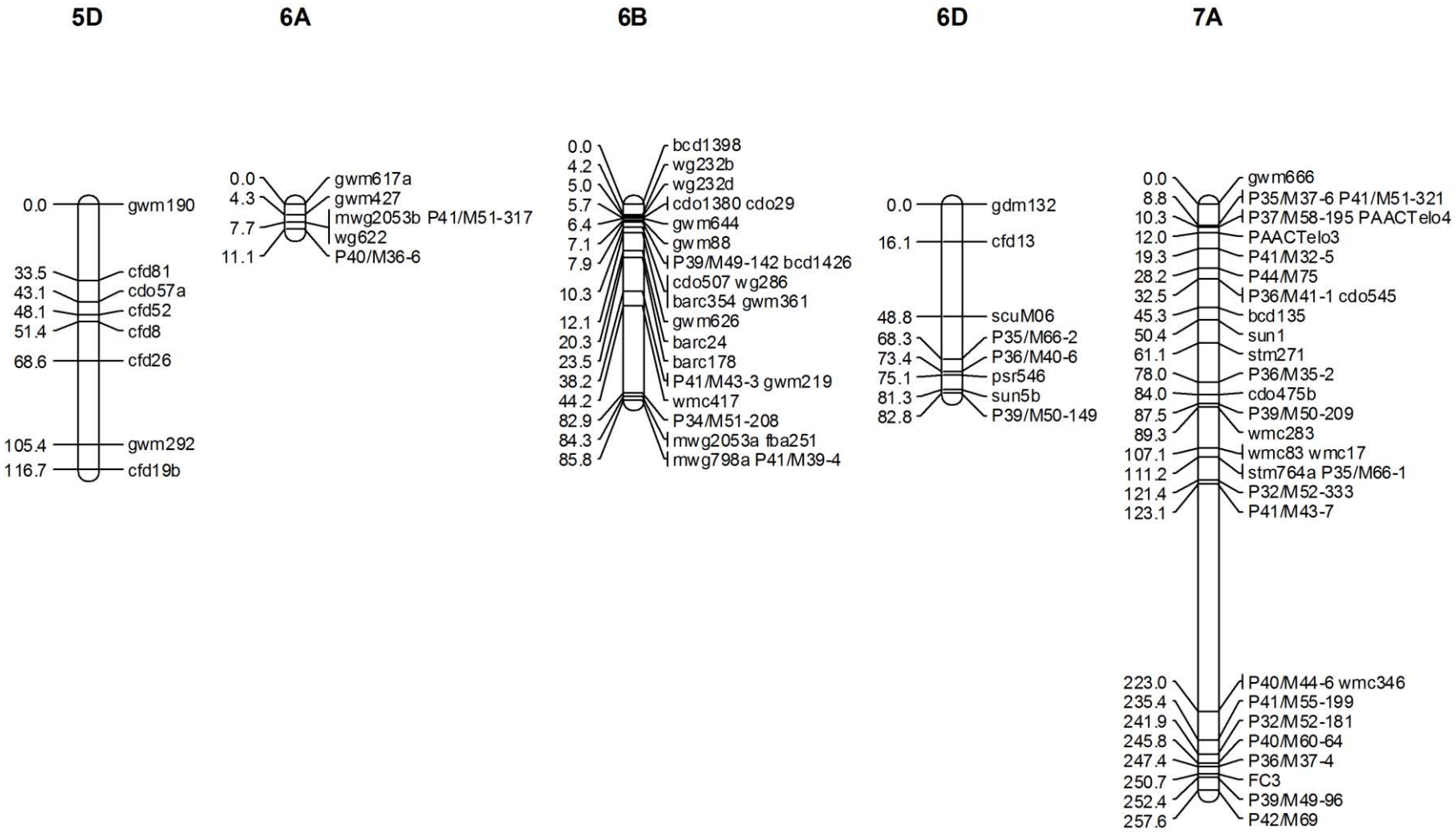
Supplemental material 6.1 Genetic map (continued)



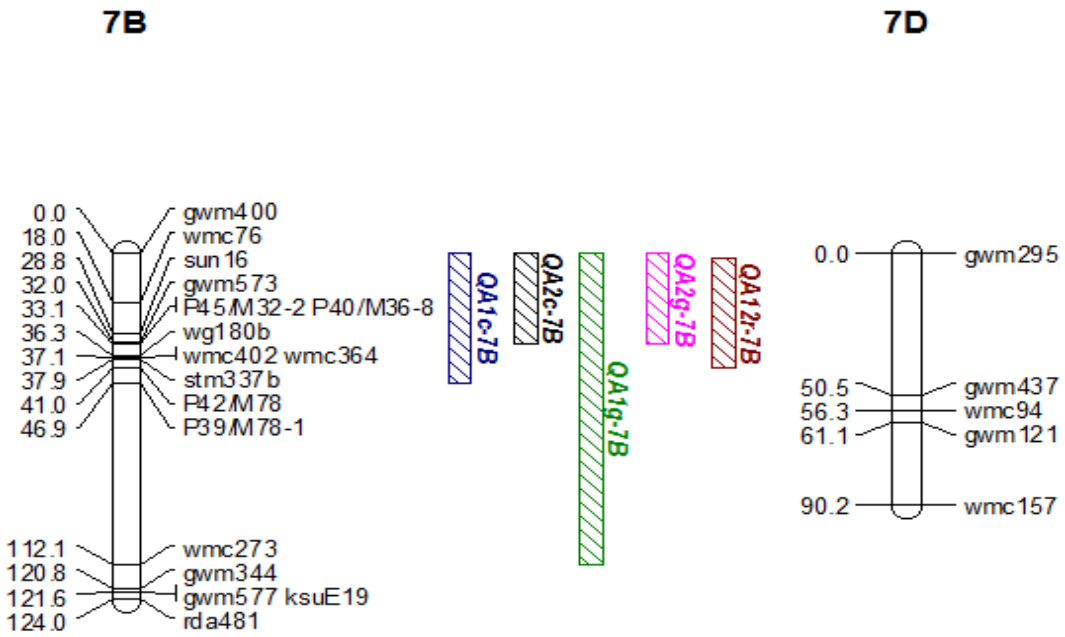
Supplemental material 6.1 Genetic map (continued)



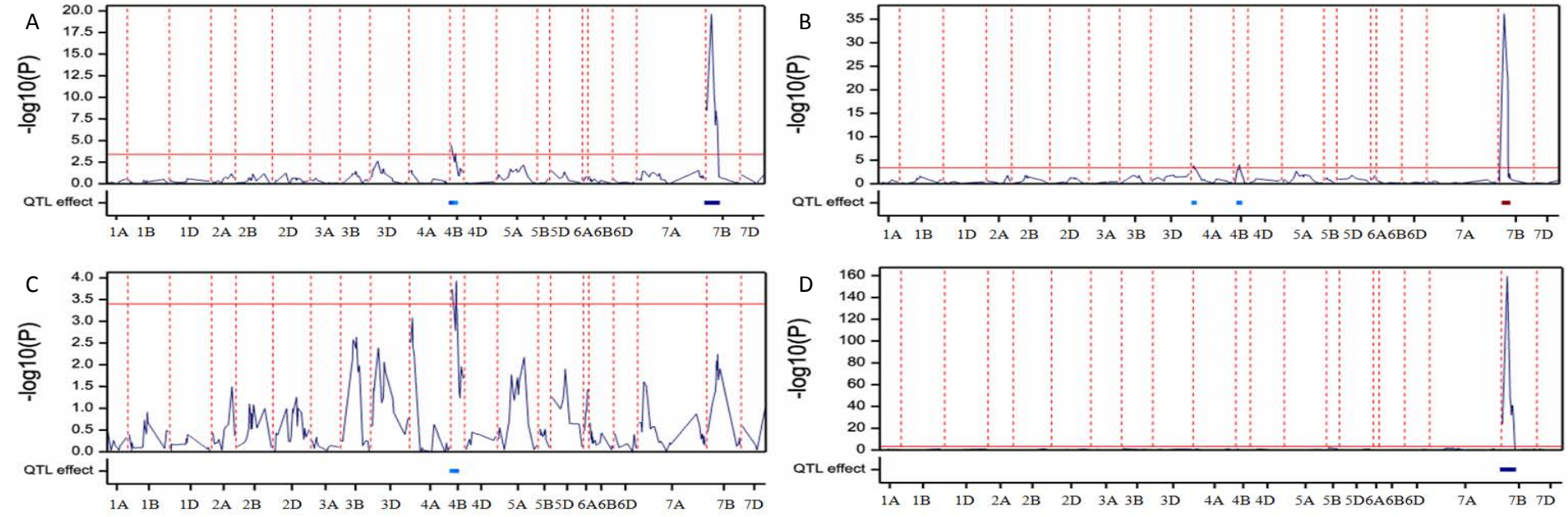
Supplemental material 6.1 Genetic map (continued)



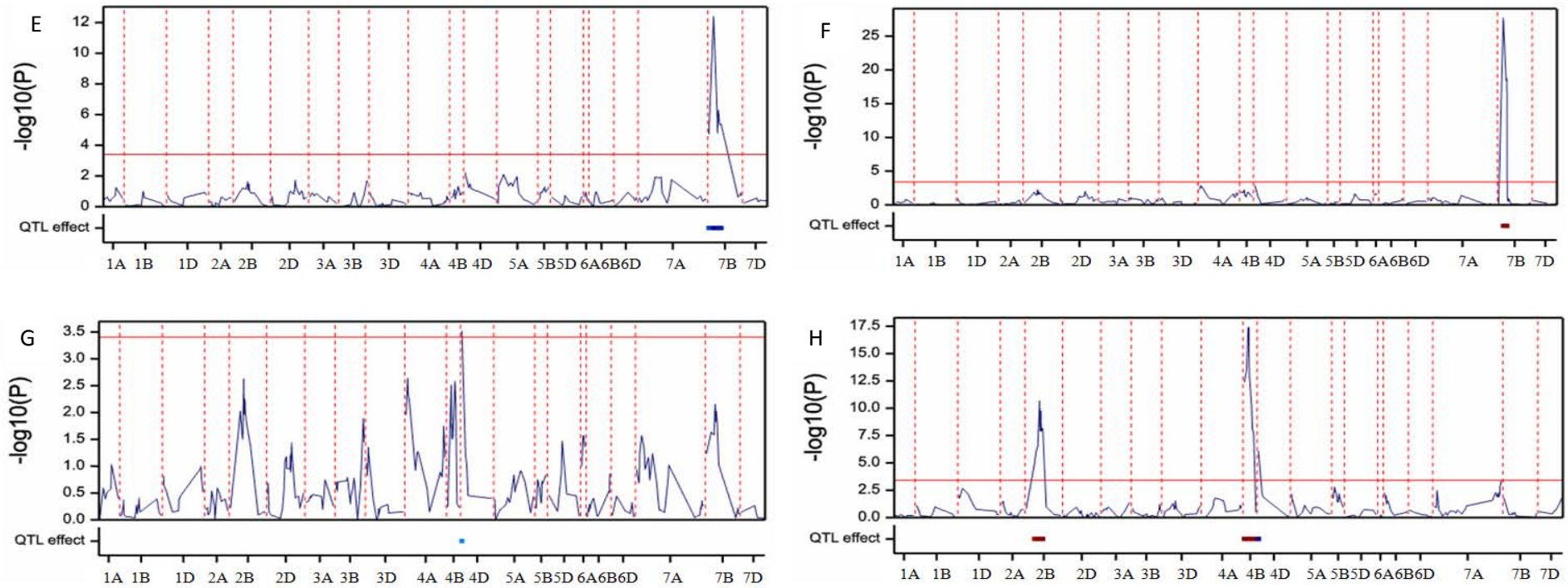
Supplemental material 6.1 Genetic map (continued)



Supplemental material 6.2 QTL for ACG traits and 100 grain weight in Sunco/Tasman doubled haploid population. QTL Test Profiles of the whole genome scan with cofactors (CIM) function in GENSTAT 14. A. QTL for ACG1 concentration (µg/g), B. QTL for ACG2 concentration (µg/g), C. Total QTL concentration (µg/g), D. QTL for ACG1/ACG2 ratio, E. QTL for ACG1 content (µg/grain), F. QTL for ACG2 content (µg/grain) G. QTL for Total ACG content (µg/grain), H. QTL for 100 grain weight (g). For the QTL effect, blue: Sunco effect, red: Tasman effect.



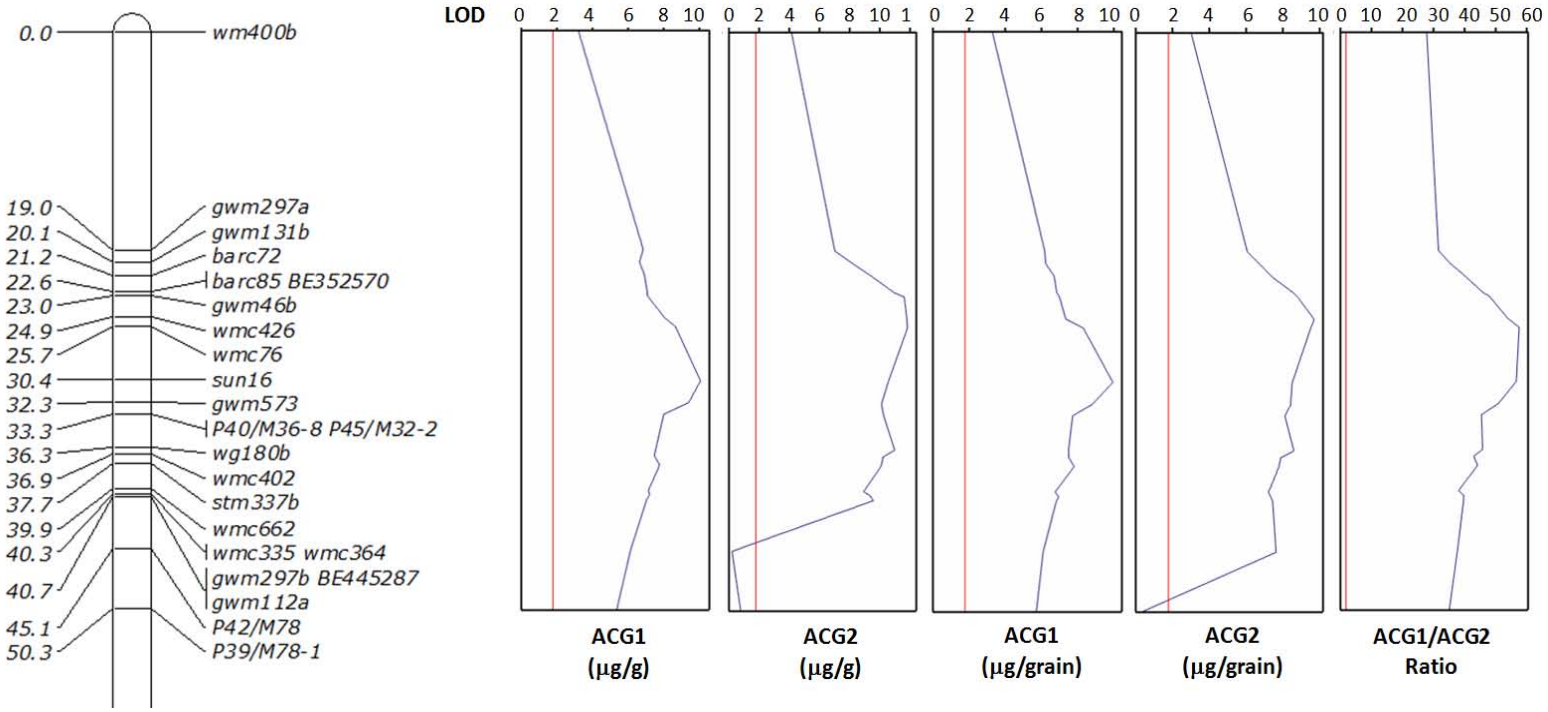
Supplemental material 6.2 Genome Scan for ACG traits in Sunco/Tasman doubled haploid population (continued).



Supplemental material 6.3 Analysis of polymorphic markers located on 7BS of Sunco/Tasman doubled haploid population; ns = not significant; *: significant ($p<0.001$, Fpr = 3.841).

Markers	$\chi^2_{1:1}$	Significance	A genotypes	B genotypes	*n*	missing data
gwm400b	3.79	ns	151	119	270	5
gwm0297a	0.09	ns	138	133	271	4
gwm0131b	0.18	ns	139	132	271	4
barc0072	0.03	ns	136	133	269	6
BE352570	0.77	ns	103	116	219	56
barc0085	0.06	ns	130	134	264	11
gwm0046b	0.30	ns	130	139	269	6
wmc0426	0.03	ns	133	136	269	6
wmc0076	0.02	ns	131	129	260	15
sun16	2.99	ns	77	57	134	141
gwm573	0.40	ns	64	57	121	154
P45/M32-2	0.55	ns	62	54	116	159
P40/M36-8	1.23	ns	75	62	137	138
wg180b	1.94	ns	83	66	149	126
wmc402	2.16	ns	84	66	150	125
stm337b	0.46	ns	73	65	138	137
wmc0662	0.00	ns	135	134	269	6
wmc0335	0.03	ns	135	132	267	8
wmc0364	0.09	ns	138	133	271	4
gwm0297b	0.00	ns	135	136	271	4
BE445287	0.03	ns	70	72	142	133
gwm0112a	0.14	ns	130	124	254	21
P42/M78	0.56	ns	77	68	145	130
P39/M78-1	0.01	ns	70	71	141	134
wmc273	2.75	ns	44	61	105	170
gwm344	2.28	ns	62	80	142	133
gwm577	0.57	ns	52	60	112	163
ksuE19	0.77	ns	60	70	130	145
rda481	1.46	ns	60	74	134	141
gwm0540	1.98	ns	124	147	271	4
gwm0276b	82.97	*	60	211	271	4
gwm0276a	17.37	*	170	101	271	4
gwm0112b	5.59	*	155	116	271	4
gwm0293b	60.64	*	70	199	269	6
gwm0016	0.50	ns	130	141	271	4
ksm0173b	3.67	ns	131	116	247	28
wmc0723	6.17	*	156	115	271	4
wmc0269a	10.27	*	162	109	271	4
wmc0338a	6.78	*	157	114	271	4
ksm0173a	12.02	*	141	97	238	37
ksm0045b	2.71	ns	122	149	271	4
wmc0435b	0.24	ns	139	132	271	4
gwm0111	19.29	*	96	168	264	11
ksm0051a	56.88	8	198	73	271	4
wmc0051	74.88	8	60	203	263	12
wmc0475	0.09	ns	137	134	271	4

Supplemental material 6.4 QTL for ACG content and composition detected on 7BS of the Sunco/Tasman doubled haploid population by Composite Interval Mapping (CIM) after the original map was saturated with more SSR markers. A. ACG1 concentration (µg/g), B. ACG1 content (µg/grain), C. ACG2 concentration (µg/g), D. ACG2 content (µg/grain) and E. Ratio of ACG1/ACG2. Red line = LOD threshold at Fpr<0.001.



Supplemental material 6.5 Protein sequences coded by genes located in the region of rice chromosome 6 that is syntenic to the 7BS QTL for ACG traits in Sunco/Tasman (Gramene databases accessed 19 January 2010).

BAC/PAC clones	Accession	Protein properties
AP005387	Q5Z6M3	Aluminum-activated malate transporter-like
	Q5Z6M4	Aluminum-activated malate transporter-like
	Q5Z6M5	Aluminum-activated malate transporter-like
	Q5Z6L5	Hydroxyproline-rich glycoprotein-like
	Q5Z6M0	NHL repeat-containing protein-like
	Q5Z6L4	Pr1-like protein
	Q5Z6K4	PRLI-interacting factor G-like
	Q5Z6M6	Putative nucellin-like aspartic protease
	Q5Z6K9	Putative potassium transporter
	Q5Z6K2	Putative flavonoid 3-O-glucosyltransferase
AP003491	Q5VQ32	Leaf senescence protein-like
	Q5VQ36	Leaf senescence protein-like
	Q5VQ37	Leaf senescence protein-like
	Q5VQ38	Leaf senescence protein-like
	Q5VQ40	Leaf senescence protein-like
	Q5Z6K4	PRLI-interacting factor G-like
	Q5VQ48	Putative amidase
	Q5VQ44	Putative bacterial blight resistance protein
	Q5Z6K2	Putative flavonoid 3-O-glucosyltransferase
AP005813	Q8H2H0	Hd1
	Q5VN15	Leaf senescence protein-like
	Q8H2I6	Leaf senescence protein-like
	Q8H2G7	Putative curly leaf protein
	Q5VN12	Putative Importin 9
	Q8H2H2	Putative peroxidase
	Q5VN06	Putative Polycomb protein EZ1
	Q5VN14	Putative signal peptidase 18K chain
	Q8H2I4	Putative signal peptidase subunit
	Q5VN11	Somatic embryogenesis receptor kinase 1-like
AP003044	Q5VR95	Armadillo/beta-catenin repeat protein-related-like
	Q5VR96	basic/helix-loop-helix 86
	Q9FDX8	Hd1
	Q5VR92	Putative adaptor protein kanadaptin
	Q9FP07	Putative curly leaf protein
	Q9FP22	Putative heat shock factor binding protein
	Q5VN12	Putative Importin 9
	Q9FNZ8	Putative Na+-dependent neutral amino acid transporter
	Q9FP19	Putative nucleolar protein family A member 2
	Q9FP11	Putative Peroxidase 49
	Q5VN06	Putative Polycomb protein EZ1
	Q9FP16	Putative polyprotein
	Q9FP13	Putative somatic embryogenesis protein kinase 1
	Q5VN11	Somatic embryogenesis receptor kinase 1-like

Supplemental material 6.5 (continued)

BAC/PAC clones	Accession	Protein properties
AP003538	Q5VPW0	Hydroxyproline-rich glycoprotein-like
	Q5VPX2	Putative resistance protein
AP003540	Q5VPU9	Carboxyl-terminal peptidase-like
	Q5VPU1	HGWP repeat containing protein-like
AP005822	Q5VMY9	Hydroxyproline-rich glycoprotein-like
	Q5VMZ3	Hydroxyproline-rich glycoprotein-like
	Q5VMZ4	Hydroxyproline-rich glycoprotein-like
	Q5VMY4	Putative DNA topoisomerase I
	Q5VN00	Putative NBS-LRR class RGA
	Q5VMY6	Putative o-methyltransferase
	Q5VMZ1	Splicing coactivator subunit-like protein
	Q5VMZ5	Zinc knuckle containing protein-like
AP005695	Q5VMY9	Hydroxyproline-rich glycoprotein-like
	Q5VN46	Putative dehydration-responsive protein RD22
	Q5VMY4	Putative DNA topoisomerase I
	Q5VMY6	Putative o-methyltransferase
	Q5VMS9	Putative UDP-glycosyltransferase
	Q5VMT1	Putative UDP-glycosyltransferase
	Q5VN44	Putative UDP-glycosyltransferase
	Q5VMZ1	Splicing coactivator subunit-like protein
AP006050	Q5VMR7	2x PHD domain containing protein-like
	Q5VMR0	Calcineurin B-like protein
	Q5VMS7	HGWP repeat containing protein-like
	Q5VMS6	HGWP repeat containing protein-like
	Q5VMR2	Hydroxyproline-rich glycoprotein-like
	Q5VMS2	Hydroxyproline-rich glycoprotein-like
	Q5VMR5	Phosphatidylinositol 3-and 4-kinase family-like
	Q5VMS8	Pr1-like protein
	Q5VMS9	Putative UDP-glycosyltransferase
	Q5VMT1	Putative UDP-glycosyltransferase
	Q5VMS1	Putative UDP-glycosyltransferase
	Q5VMS0	Putative UDP-glycosyltransferase
	Q5VMS3	UDP-glycosyltransferase-like
	Q5VMS5	Zinc knuckle containing protein-like
	Q5VMS4	Zinc knuckle containing protein-like
AP004791	Q5VNE3	BRUSHY1-like
	Q5VMR0	Calcineurin B-like protein
	Q5VNF6	Endosperm specific protein-like
	Q5VNF4	HAP3 transcriptional-activator
	Q5VMR2	Hydroxyproline-rich glycoprotein-like
	Q5VNE7	Methyl-CpG binding protein-like
	Q5VNF3	Patatin-like phospholipase domain containing protein-like
	Q5VNG4	Putative auxin-independent growth promoter
	Q5VNE4	Putative dof zinc finger protein
	Q5VNF2	Putative lectin-like receptor kinase
	Q5VNF8	Putative TMV-MP30 binding protein 2C

Supplemental material 6.5 (continued)

BAC/PAC clones	Accession	Protein properties
AP005830	Q6YVE7	Mitochondrial aspartate-glutamate carrier protein-like
	Q6YVD9	Peptidase family-like protein
AP005659	Q5VN64	NBS-LRR disease resistance protein-like
	Q5VMI1	Putative brassinosteroid insensitive 1
	Q5VN67	Putative NBS-LRR disease resistance protein
	Q5VN70	Putative NBS-LRR disease resistance protein
	Q5VN78	Putative NBS-LRR disease resistance protein
	Q5VN81	Putative NBS-LRR disease resistance protein
	Q5VN82	Putative nitrate-induced NOI protein
	Q5VMI0	*C* -glycosyltransferase (*OsCGT*)
	Q5VN75	Subtilase-like protein
AP006584	Q5VMG6	: Epstein-Barr virus EBNA-1-like protein
	Q5VMH2	: HGWP repeat containing protein-like
	Q5VMH1	: Pr1-like protein
	Q5VMH7	: Pr1-like protein
	Q5VMI1	: Putative brassinosteroid insensitive 1
	Q5VMI0	: Putative UDP-glycosyltransferase
	Q5VMG8	: Putative UTP-glucose glucosyltransferase
	Q5VMH3	: Zinc knuckle containing protein-like
AP006860	Q5VMD9	: Cell wall protein-like
	Q5Z577	: Embryogenesis transmembrane protein-like
	Q5Z580	: Embryogenesis transmembrane protein-like
	Q5Z590	: Hydroxyproline-rich glycoprotein-like
	Q5Z575	: Putative embryogenesis transmembrane protein
	Q5Z589	: Putative glucosyltransferase-3
	Q5VME5	: Putative UDP-glycosyltransferase
	Q5Z588	: Root cap protein 1-like
	Q5VMD8	: Zinc finger protein-like
AP005761	Q5Z569	Aminotransferase-like protein
	Q5Z577	Embryogenesis transmembrane protein-like
	Q5Z580	Embryogenesis transmembrane protein-like
	Q5Z570	Embryogenesis transmembrane protein-like
	Q5Z567	Embryogenesis transmembrane protein-like
	Q5Z590	Hydroxyproline-rich glycoprotein-like
	Q5Z575	Putative embryogenesis transmembrane protein
	Q5Z589	Putative glucosyltransferase-3
	Q5Z588	Root cap protein 1-like

Supplemental material 6.6 Protein sequences coded by genes located in the region of rice chromosome 8 that is syntenic to the 7BS QTL for ACG traits in Sunco/Tasman (Gramene databases accessed 19 January 2010).

BAC/PAC clones	Accession	Protein properties
AP005245	Q6Z3T8	Auxin-regulated protein-like
	Q6Z3R2	Cyt-P450 monooxygenase
	Q6Z3T7	Epstein-Barr virus EBNA-1-like protein
	Q6Z3T3	Putative 60S ribosomal protein L31
	Q6Z3T9	Putative DNA-damage-repair/toleration protein
	Q6Z3S1	Putative leucine-rich repeat/receptor protein kinase
	Q6Z3R6	Putative transcription factor RAU1
	Q6Z3R9	RRNA methylase-like
AP005254	Q6Z3F0	Cyt-P450 monooxygenase
	Q6Z3R2	Cyt-P450 monooxygenase
	Q6YVS8	N-acetyltransferase and Transcription factor-like protein
	Q6Z3D6	Putative Cyt-P450 monooxygenase
	Q6YVT1	Putative ethylene-insensitive protein
	Q6YVS9	Splicing coactivator subunit-like protein
AP005816	Q6YVT6	Circumsporozoite protein-like
	Q6Z3F0	Cyt-P450 monooxygenase
	Q6YVT0	Lipoxygenase, chloroplast
	Q7EXZ6	Lipoxygenase-like protein
	Q6YVS8	N-acetyltransferase and Transcription factor-like protein
	Q7EXZ4	Putative beta-glucosidase isozyme 2
	Q7EXZ5	Putative beta-glucosidase isozyme 2
	Q6YVT1	Putative ethylene-insensitive protein
	Q7EXZ2	Putative SBP-domain protein
	Q6YVS9	Splicing coactivator subunit-like protein
AP006049	Q84YJ6	DNA-binding protein family-like
	Q7EXZ6	Lipoxygenase-like protein
	Q84YK8	Probable lipoxygenase 8, chloroplast precursor
	Q84YJ8	Putative alcohol dehydrogenase homolog
	Q7EXZ4	Putative beta-glucosidase isozyme 2
	Q7EXZ5	Putative beta-glucosidase isozyme 2
	Q84YK7	Putative beta-glucosidase isozyme 2
	Q84YK6	Putative beta-glucosidase isozyme 2 precursor
	Q84YJ1	Putative cup-shaped cotyledon
	Q7EXZ0	Putative floral organ regulator
	Q84YJ9	Putative high-affinity potassium transporter
	Q7EXZ2	Putative SBP-domain protein
	Q84YK4	Putative SBP-domain protein 5
	Q7EXY6	Selenium binding protein-like protein
AP004765	Q6Z8N5	3-hydroxy-3-methylglutaryl-coenzyme A reductase 3
	Q6Z8M4	Lectin-like protein kinase-like
	Q7EZ92	Myosin heavy chain-like protein
	Q6Z8N6	Protein cdc2 kinase

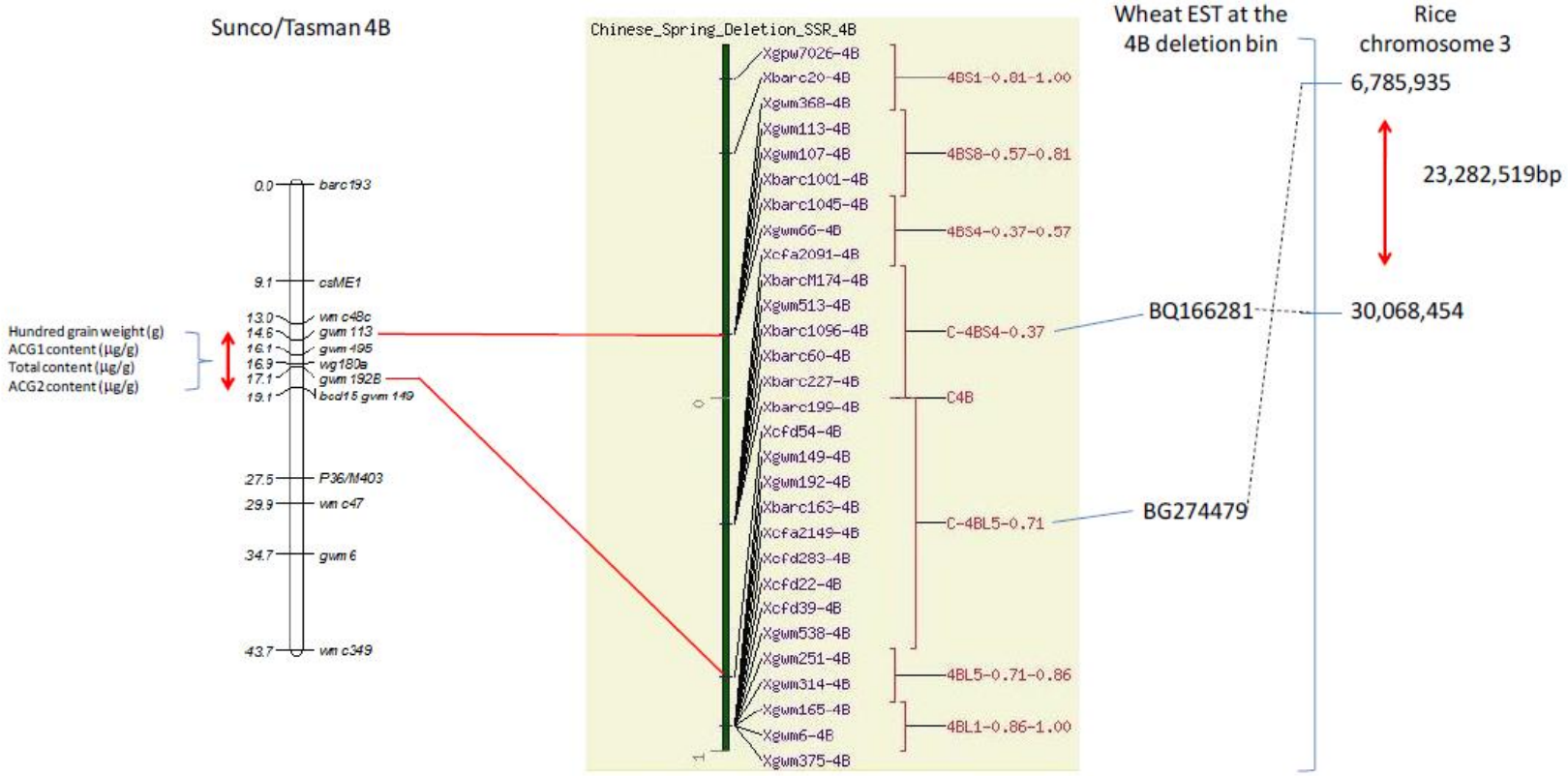
Supplemental material 6.6 (continued)

BAC/PAC clones	Accession	Protein properties
AP004765	Q6Z8P2	Putative aminoacylase
	Q6Z8N8	Putative AT-hook DNA-binding protein
	Q6Z8N9	Putative AT-hook DNA-binding protein
	Q6Z8M5	Putative lectin-like protein kinase
	Q6Z8N3	Putative phosphoprotein phosphatase PP7
	Q6Z8M9	Putative proton pump interactor
	Q6Z8P4	Putative PSR9
	Q6Z8L7	Putative Rab geranylgeranyltransferase, beta subunit
	Q6Z8M8	Putative SPL1-Related2 protein
	Q6Z8N7	Thylakoid lumen protein, chloroplast-like
AP004761	Q7EZ92	Myosin heavy chain-like protein
	Q7EZ93	Putative C2H2 zinc-finger protein
	Q84JI5	Putative C2H2 zinc-finger protein
	Q7EZ84	Putative calcium-transporting ATPase 8, plasma membrane-type
	Q84JY7	Putative calcium-transporting ATPase 8, plasma membrane-type
	Q84Z61	Putative dihydroflavonol 4-reductase
	Q7EZ87	Putative dihydroflavonol reductase
	Q84Z70	Putative flavonol 4'-sulfotransferase
	Q84Z52	TDPOZ4-like
	Q7EZ88	Ternary complex factor-like
AP004708	Q84Z89	30S ribosomal protein S16-like
	Q7EZA8	Myosin heavy chain-like protein
	Q7EZA6	Oxidase-like
	Q7EZA7	Oxidase-like
	Q7EZA3	Oxysterol-binding protein-like
	Q84Z91	Oxysterol-binding protein-like
	Q7EZ93	Putative C2H2 zinc-finger protein
	Q84JI5	Putative C2H2 zinc-finger protein
	Q7EZ84	Putative calcium-transporting ATPase 8, plasma membrane-type
	Q84JY7	Putative calcium-transporting ATPase 8, plasma membrane-type
	Q7EZB2	Putative chitinase
	Q84Z87	Putative microtubule-associated protein
	Q84Z90	Putative pathogenesis-related protein
	Q7EZB0	Putative serine/threonine-specific receptor protein kinase
AP004622	Q6YZW0	Auxin response factor 7b
	Q7EZA8	Myosin heavy chain-like protein
	Q7EZA6	Oxidase-like
	Q7EZA7	Oxidase-like
	Q6YZW3	Pentatricopeptide (PPR) repeat-containing protein-like
	Q7EZB2	Putative chitinase
	Q6YZW7	Putative leucine zipper-containing protein
	Q6YZW6	Putative mitochondrial energy transfer protein
	Q6YZW2	Putative oligouridylate binding protein
	Q6YZW8	Putative pumilio-family RNA-binding domain-containing protein

Supplemental material 6.6 (continued)

BAC/PAC clones	Accession	Protein properties
AP004622	Q6YZW4	Putative ribosomal large subunit pseudouridine synthase C protein
	Q7EZB0	Putative serine/threonine-specific receptor protein kinase
	Q6ZBK6	Putative stress related-like protein interactor
AP005509	Q6YZW0	Auxin response factor 7b
	Q7F1K3	P53 binding protein-like
	Q7F1K4	P53 binding protein-like
	Q7F1K5	P53 binding protein-like
	Q6YZW3	Pentatricopeptide (PPR) repeat-containing protein-like
	Q6YZW7	Putative leucine zipper-containing protein
	Q6YZW6	Putative mitochondrial energy transfer protein
	Q6YZW2	Putative oligouridylate binding protein
	Q6YZW8	Putative pumilio-family RNA-binding domain-containing protein
	Q7F1K6	Putative receptor-like protein kinase
	Q6YZW4	Putative ribosomal large subunit pseudouridine synthase C protein
AP003887	Q7F1J8	DnaJ protein family-like
	Q7F1F1	Putative auxin-induced protein
	Q6ZK18	Putative inositol 1,4,5-trisphosphate 5-phosphatase
	Q7F1J9	Putative L-ascorbate peroxidase
	Q6ZK21	Putative signal recognition particle receptor beta subunit
	Q7F1J2	Putative speckle-type POZ protein
	Q7F1J5	Putative speckle-type POZ protein
	Q7F1J7	Putative speckle-type POZ protein
	Q7F1J1	Zinc finger POZ domain protein-like
	Q7F1J3	Zinc finger POZ domain protein-like
AP003888	Q84QW1	basic/helix-loop-helix 91
	Q7F1E6	Pentatricopeptide (PPR) repeat-containing protein-like
	Q7F1F2	Putative 70 kDa peptidylprolyl isomerase
	Q84QV6	Putative 70 kDa peptidylprolyl isomerase
	Q7F1E7	Putative amino acid permease
	Q7F1F1	Putative auxin-induced protein
	Q84QW4	Putative auxin-induced protein
	Q84QV4	Putative beta-1,3-glucanase
	Q84QW0	Putative GTP-binding protein
	Q84QW3	Putative ribosomal protein L32
	Q84QV1	Putative type 1 capsule synthesis gene
	Q84QV2	Receptor-like protein kinase-like

Supplemental material 6.7 Synteny of Sunco/Tasman doubled haploid chromosome 4B region harbouring minor QTL for total ACG1 and ACG2 content with the physical maps of Chinese Spring wheat and rice chromosome 3.



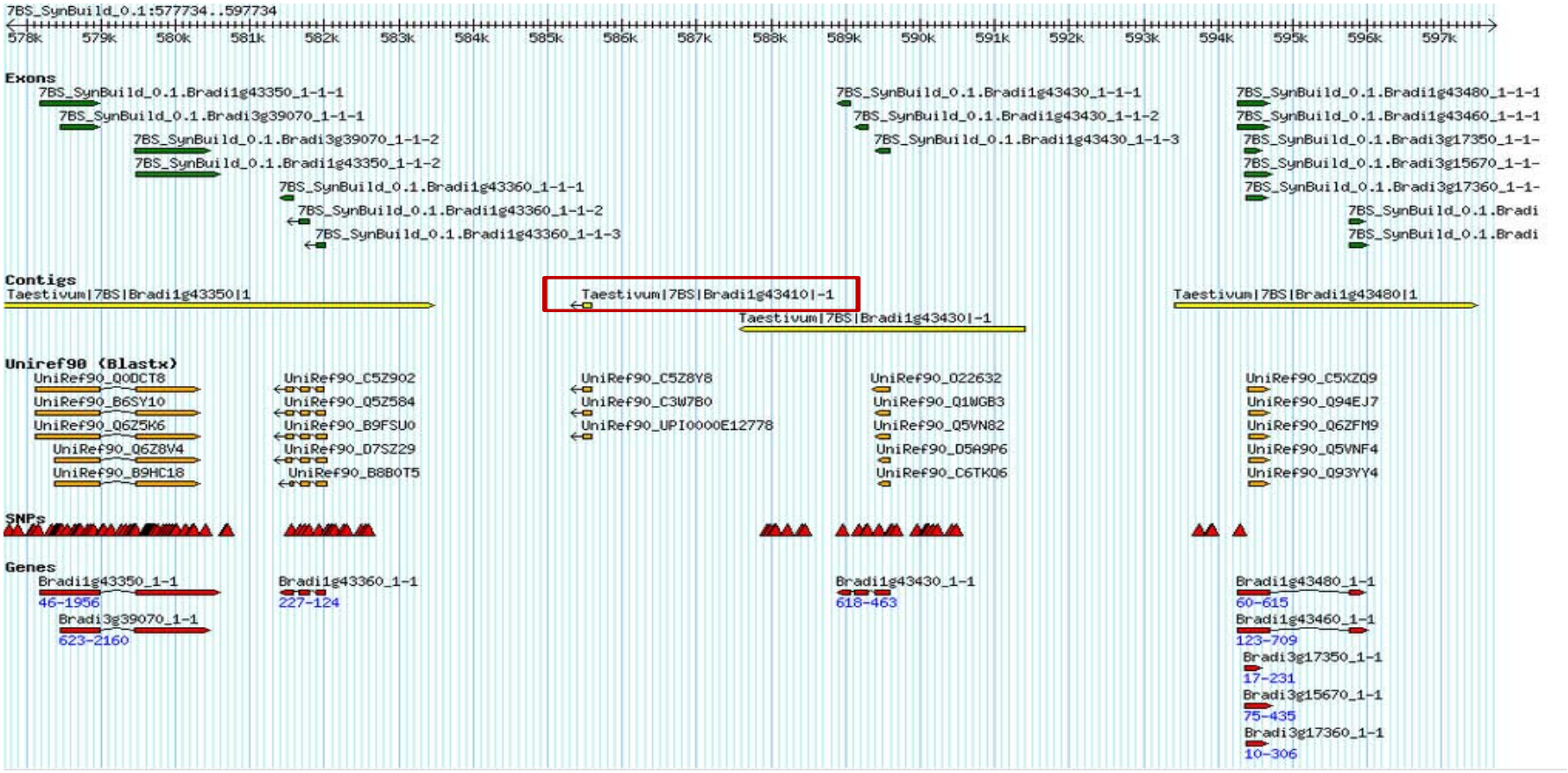
Supplemental material 6.8 Wheat 3B sequences that show interrupted synteny of wheat 7BS and rice 6 (Gramene, access 27 April 2012).

wheat 3B EST	Wheat Accession	Position	Rice 6 marker from physical map	Location		Gene locus	Gene function
				Start	Stop		
BE442681	dvo2010a-6D-BE442681	23,674,818.00 bp	Ta.21873	6,019,439.00 bp	6,019,913.00 bp	LOC_Os06g11410	cyclin
			TC254600				
			TA81015_4565	6,019,836.00 bp			
BF482491	dvo2010a-3D-BF482491	28,369,221.00 bp	PUT-153a-Triticum_aestivum-28532	2,898,072.00 bp	2,898,614.00 bp	LOC_Os06g06260	GDSL-like lipase/acylhydrolase
						LOC_Os06g06270.1	Transcription elongation factor Elf1 like
AW506610-3B	pae2008a-3B-260	98,898,079.00 bp	PUT-157a-Zea_mays-025599	15,133,732.00 bp	15,133,764.00 bp	LOC_Os06g25880	retrotransposon protein
						LOC_Os06g25890	retrotransposon protein
BF475065-3B	pae2008a-3B-284	111,662,487.00 bp	Ta.13866	7,497,601.00 bp	7,497,687.00 bp	LOC_Os06g13580	expressed protein, unidentified
BJ233340-3B	pae2008a-3B-1153	445,912,012.00 bp	TA75771_4565	17,539,142.00 bp	17,539,356.00 bp	LOC_Os06g30370	osMFT1 MFT-Like1 homologous to Mother of FT and TFL1 gene; contains Pfam profile PF01161: Phosphatidylethanolamine-binding
			PUT-153a-Triticum_aestivum-20991				
			TC265741				
			Ta.10074	17,539,154.00 bp			
BG263580-3B	pae2008a-3B-1857	673,967,555bp	PUT-153a-Triticum_aestivum-20993	16,876,437.00 bp	16,876,514.00 bp	LOC_Os06g29450	expressed protein, unidentified
			TA96955_4565			LOC_Os06g29460	expressed protein, unidentified

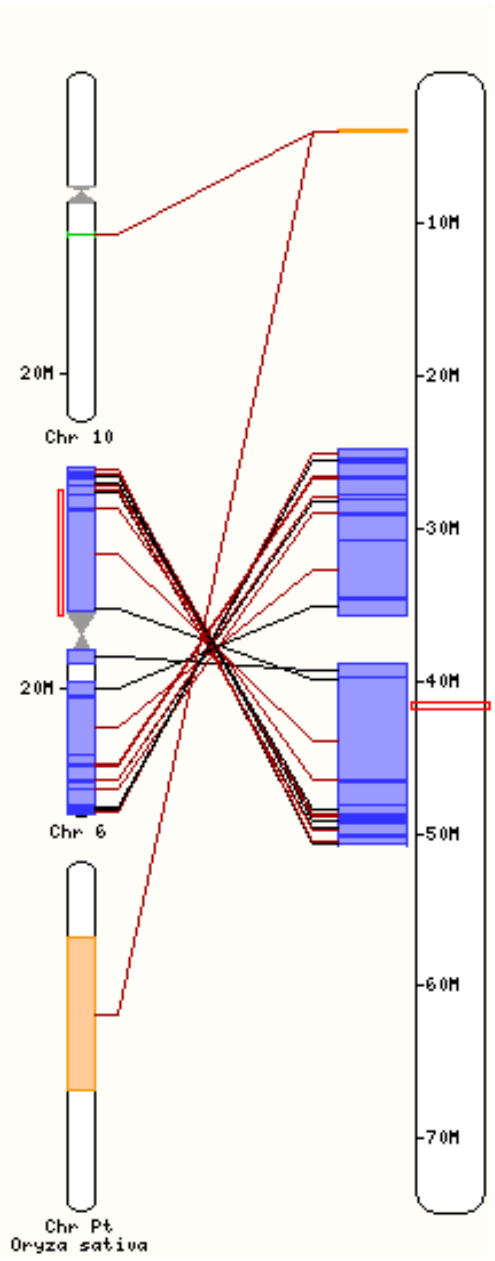
Supplemental material 6.8 (continue)

wheat 3B EST	Wheat Accession	Position	Rice 6 marker from physical map	Location		Gene locus	Gene function
				Start	Stop		
BF485490-3B	pae2008a-3B-2083	729,556,473.00 bp	TA86527_4565	7,550,588.00 bp	7,748,466.00 bp	LOC_Os06g13640	expressed protein, unidentified
			PUT-153a-Triticum_aestivum-16512			LOC_Os06g13650	alpha-mannosidase 2
			TC238943			LOC_Os06g13660	alanyl-tRNA synthetase
						LOC_Os06g13670	E2F family transcription factor
						LOC_Os06g13680	B12D protein
						LOC_Os06g13690	hypothetical protein, unknown
						LOC_Os06g13700	hypothetical protein, unknown
						LOC_Os06g13710	glycosyltransferase
						LOC_Os06g13720	dehydrogenase E1 component domain containing prot
						LOC_Os06g13730	glutamate receptor precursor
						LOC_Os06g13740	transposon protein
						LOC_Os06g13750	expressed protein, unidentified
						LOC_Os06g13760	glycosyl transferase 8 domain containing protein
						LOC_Os06g13770	transposon protein
						LOC_Os06g13780	expressed protein, unidentified
						LOC_Os06g13789	hypothetical protein, unknown
						LOC_Os06g13800	conserved hypothetical protein
						LOC_Os06g13810	pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit beta
						LOC_Os06g13820	dynamin
						LOC_Os06g13830	endoglucanase
						LOC_Os06g13839	RNA recognition motif containing protein
						LOC_Os06g13850	OsFBX193 - F-box domain containing protein
						LOC_Os06g13860	RCD1
						LOC_Os06g13870	U-box protein CMPG1
						LOC_Os06g13880	expressed protein, unidentified
						LOC_Os06g13890	conserved hypothetical protein
						LOC_Os06g13900	transposon protein
						LOC_Os06g13910	hypothetical protein, unknown

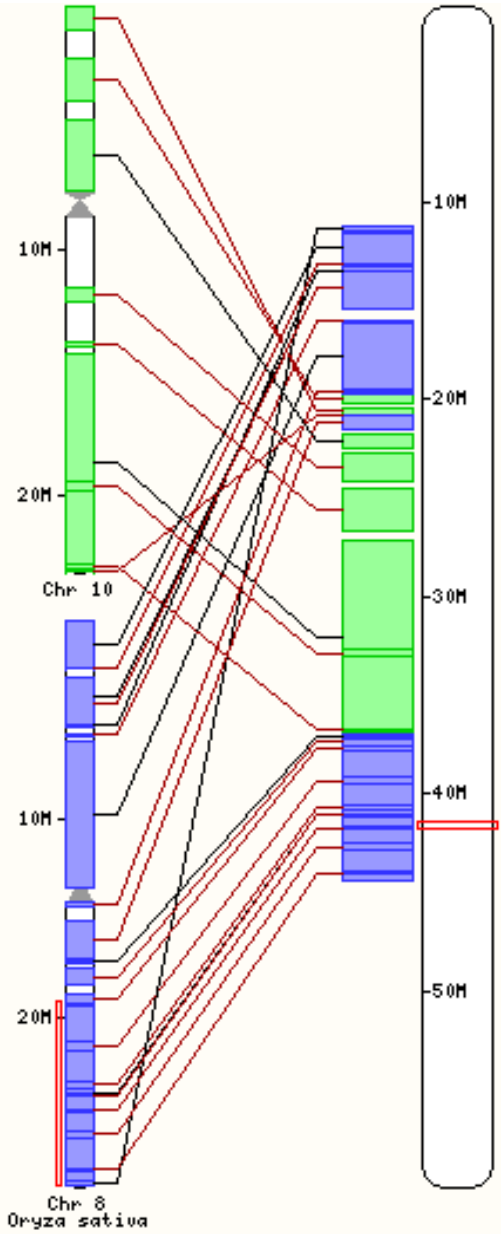
Supplemental material 6.9 Synteny between 7BS of wheat and Brachypodium distachyon at BRADIG43410 locus (115bp) (red box). Genome Browser for 7BS_Syn_Build_0.1:577734..597734 was taken from www.wheatgenome.info/.



Supplemental material 6.10 Synteny block between *Brachypodium distachyon* chromosome 1 and rice chromosomes 6 and 10. Illustration was taken from Gramene database (accessed 11 March 2012). Blocks in blue colour are synteny block between rice chromosme 6 and the *Brachypodium* chromsome 1, while the large white chromosome is the *Brachypodium distachyon* chromosome 1. Small red rectangle in the *Brachypodium distachyon* chromosome shows the location of syntenic SNP marker *BE352570*.



Supplemental material 6.11 Synteny block between *Brachypodium distachyon* chromosome 3 and rice chromosomes 8 and 10. Illustration was taken from Gramene database (accessed 11 March 2012). Blocks in blue colour are synteny block between rice chromosme 8 and *Brachpodium* chromosome 3, while the large white chromosome is the *Brachypodium distachyon* chromosome 3. Small red rectangle in the *Brachypodium distachyon* chromosome shows the location of sythenic SNP marker *BE445287*.



Supplemental material 6.12 Multiple alignments of the UDP-glycosyltransferases from the region of rice chromosome 6 than is syntenic with the Ratio QTL on 7BS of Sunco/Tasman with other UDP-glycosyltransferases. The sequences are similar to that shown in Figure 14. Secondary structure conservation shown by pink (α-helix) and yellow (β-helix) shades. The red arrows shows His-33 and Asp-163 residues that are catalytically important for interaction with UDP-sugar, while the blue arrow shows the single amino acid polymorphism between the putative *O*-glycosyltransferases and *C*-glycosyltransferases. The alignment is available on the next page.


N-terminal domain
Nβ1
loopN1
Nα1
Nβ2
Nα2
Nβ3
loopN3
Nα3
Nβ4
loopN4
Nα4
Nβ5
loopN5
Nα5
loopN5a
Nα5
loopN5b
Nα5b
Nβ6
Nα6
Nβ7
interdomain linker
C-terminal domain
Cα0
Cβ1
Loop C1
Cα1
Cβ2
Cα2

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