University of Southern Queensland

Use of Marker Assisted Selection for the Introgression of Quality Traits from Australian into Chinese Wheats

A Dissertation Submitted by

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Abstract

Quantitative Trait Loci **(**QTLs) for polyphenol oxidase and xanthophyll have a significant impact on variation in wheat flour for noodle colour and colour stability. QTLs from two Australian wheat cultivars, Sunco and Tasman, have been backcrossed into two Chinese wheat varieties, Chuanmai 22 and Mianyang 11, to assess marker predictability for these important traits in significantly different genetic backgrounds. The concept of Marker-Assisted-Selection (MAS) is being trialled in this study as a proposed method for wheat improvement. In this approach molecular markers are used in conjunction with backcross breeding methods to introgress specific characters into elite breeding materials, with the goal of improving the quality attributes of wheat for the Asian noodle market. After three single seed descent generations, the backcross populations generated allow four QTLs to be investigated. These include two for polyphenol oxidase (chromosome 2A and 2D) and two for xanthophyll (chromosome 3B and 7A).

This research was successful in identifying microsatellite markers that are capable of predicting PPO activity levels and Xanthophyll content within the backcross populations. These microsatellites were validated as useful

markers for these quality traits, as they have also found to be important in the Sunco x Tasman doubled haploid population. The combination of marker assisted selection and backcrossing has generated three lines that contain different combinations of the PPO activity and Xanthophyll content QTLs. These lines have been found to produce low levels of PPO activity and have a low Xanthophyll content. This improvement in flour colour and colour stability highlights the potential of marker assisted selection as a useful tool in wheat breeding.

Declaration

I certify that the ideas, experimental work, results, analyses and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

…… Date:

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ENDORSEMENT

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Signature of Supervisor

Professor Mark Sutherland

Previously Published Material

Some of this work has been previously presented at scientific conferences.

Cavallaro, B., Storlie, E., Sutherland, M.W., Mares, D., Sheppard, J., & Banks, P., (2004) Backcrossing QTLs from Australian to Chinese wheats, Wheat Breeding Society of Australia, 11th Assembly, Canberra ACT.

Acknowledgements

While working as a Research Assistant I commenced this master's project part-time in 2002. Over the following six years a few major events occurred that significantly slowed the completion of this master's thesis. I was married in the end of 2004, so that warranted a little holiday. But it was in March of 2006 that my study life was successfully slowed to a halt by the arrival of our son, Noah. At this time I took a leave of absence from study and concentrated on the important job of being a new Mum. The final distraction was during the first six months of 2008 when we all travelled to Oxford, UK where my husband, Dr Mike Watson, spent a sabbatical leave.

It has been an eventful seven years after deciding to commence this master's project. And it wouldn't have been possible without the support and guidance from many people along the way.

This project came about through a large Australian Centre for International Agricultural Research (ACIAR) funded project entitled "Wheat Improvement in Sichuan Province" (1999-2006) on which I was employed as a research assistant. This large project coordinated by Mark Sutherland at USQ, was based on a close collaboration with the Crop Research Institute within the Sichuan Academy of Agricultural Sciences (SAAS), located in Chengdu, China, the Leslie Research Centre (QDPI & F), the University of

Sydney, Cobbitty and the University of Adelaide, Waite Campus. I would like to thank all involved in this overall project as they have supported me during the time I was employed, but also while I completed my study.

During this project there were a number of trips to China to meet with our colleagues from Sichuan. I would especially like to thank Dr Yuchun Zou for his hospitality, but most of all for detailed knowledge of the wheat varieties used in this master's project, and all the information that I gained from him throughout these last years.

The initial concept for this project was developed by Dr. Eric Storlie while he was the Project Officer for the ACIAR project employed at USQ. It was he who made the initial crosses to establish the four wheat populations which are central to this Masters project. A field trial planted at the Leslie Research Centre in 2005 was also an integral part of this research. This trial was maintained by Lloyd Mason (Leslie Research Centre) and without his help and hard work the trial would not have been such a success. I would like to thank a number of the staff at the Leslie Research Centre, these being David Martin and all the Quality Laboratory staff, and Mandy Christopher and Raeleen Jennings of the Molecular Marker Laboratory.

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Chapter 1. Introduction and Literature Review

1.0 Introduction

"Plant breeding is essentially an election made by man of the best plants within a variable population as a potential cultivar" (Sanchez-Monge 1993). The selection and cultivation of grains for food has been a constant effort by man for centuries. It is believed that wheat originated in south-western Asia, and wild cereals can still be located within their natural and original habitats in the foot hills of Iraq-Kurdistan and southeast Turkey (Feldman 2001). The cultivation of wheat occurred 10,300 – 6,200 years ago. Over this large period of time, basic but very important characteristics would have influenced the selection of wheat plants. These included: plants with large grains that didn't shatter or lodge; and having the ability to survive through changes in environmental conditions. These selections paved the way for modern wheat breeding.

During the 19th century researchers focused on the origins of wheat and its genetic background with the discovery of the work of Mendel. The onset of what is termed "Modern Plant Breeding" has increased the understanding of all aspects of plant/wheat breeding with respect to its genetics, physiology, pathology and chemistry. Knowledge of these processes and the mechanisms involved has allowed breeders to regulate and increase the variability needed to produce new varieties (Feldman 2001).

Demand on wheat breeding has become even more important with the increasing requirement for global food production. However, conventional breeding methods are time consuming taking up to twelve years for the release of new varieties (Korzun 2002). It has been reported that using a combination of traditional breeding methods with current diverse molecular techniques could help to reduce the time for release and offer a reliable improvement of elite breeding material (Liu *et al.* 2004, Sorrells and Wilson 1997).

1.1 Wheat Production

Wheat is a major stable food and is grown widely in many countries of the world. It has been reported that by 2020 the majority of the world's wheat consumption will be in developing countries and this will have a direct affect on the amount of wheat needing to be imported (CommodityOnline 2008).

The world wheat production is approximately 585 million tons per annum, and the major producers are the European Union (EU-27), which is comprised of 27 countries, China, India, Russia and USA (CommodityOnline 2008). The Australian Bureau of Agricultural and Resource Economics (ABARE) have produced economic data on the production and consumption for Australia and the major world producers for a number of years and seasons (Table 1-1) (ABARE 2008).

Table 1-1: Total world production of wheat, and wheat production by major wheat producers: China, EU27, India, Russia and USA.

	2006-07 (Mt)	2007-08 (Mt)	2008-09 forecast (Mt)
World Production	**598**	**609**	**672**
EU27	125	120	146
China	109	110	113
India	69	76	78
United States	49	56	67
Russian Federation	45	49	55
Consumption	612	612	643
- human	442	446	451
- feed	98	87	111
Price	US$ 212 /t	US$ 362 /t	US$ 325 /t

(Data source: ABARE 2008)

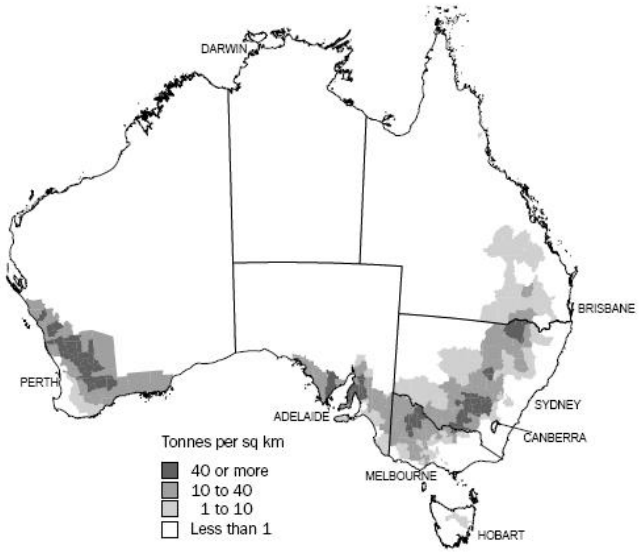
For the purpose of this chapter the emphasis on wheat production will be on the countries Australia and China as they have direct impact on this study.

1.1.1 *Wheat Production in Australia*

In Australia wheat is a winter grown crop that is sown between May and July and is harvested in late spring/early summer. The amount of wheat produced throughout Australia differs for each state with respect to yield and cropping area. What is termed the Australian Wheat Belt spans most states with the exception of the Northern Territory (Figure 1-1). A range of white-grained wheat varieties are grown in Australia including bread wheats and durum wheats (for pasta) (Christopher and Banks 2002). Soft grained wheat varieties grown in the Australian wheat belt are concentrated in Western Australia and are generally used for biscuits and noodle production. The varieties planted vary throughout Australia and are determined by

their adaptation for particular environments, resistance to diseases and their tolerance to drought and frost.

Figure 1-1: Areas of wheat production in Australia. Data from the Agricultural Census of 2005-06 by the Australian Bureau of Statistics *(Data source: http://www.abs.gov.au)*



There is a noticeable difference in the wheat production and cropping areas between the states of Australia, and it is Western Australia that is the highest producer of the entire Australian wheat belt (Figure 1-2 and 1-3).

Figure 1-2: Australian wheat production (kt) of six states over the last five years (2002-2007), and the predicted season for 2008-09 *(Data source: ABARE 2008)*

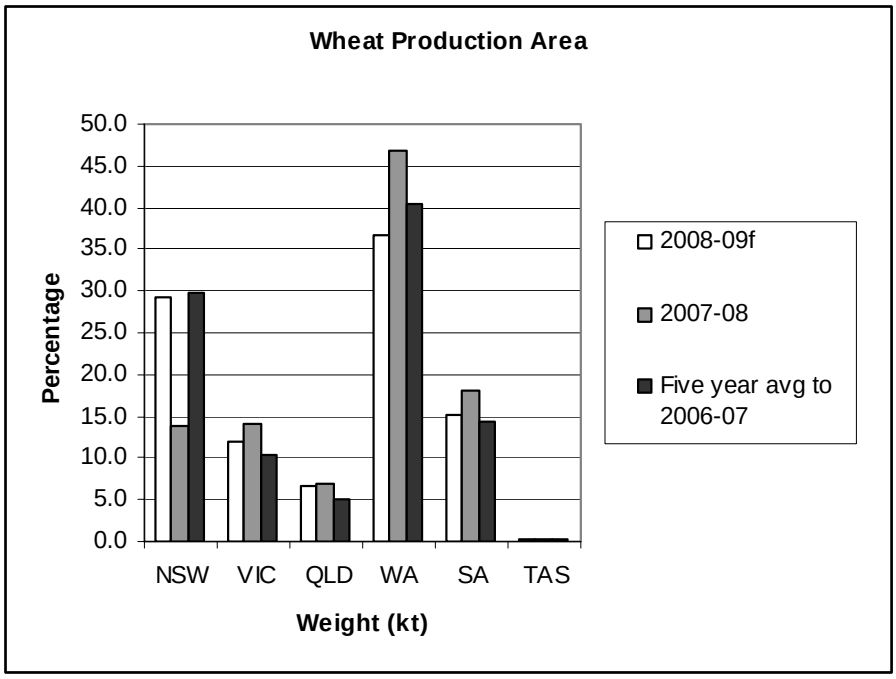
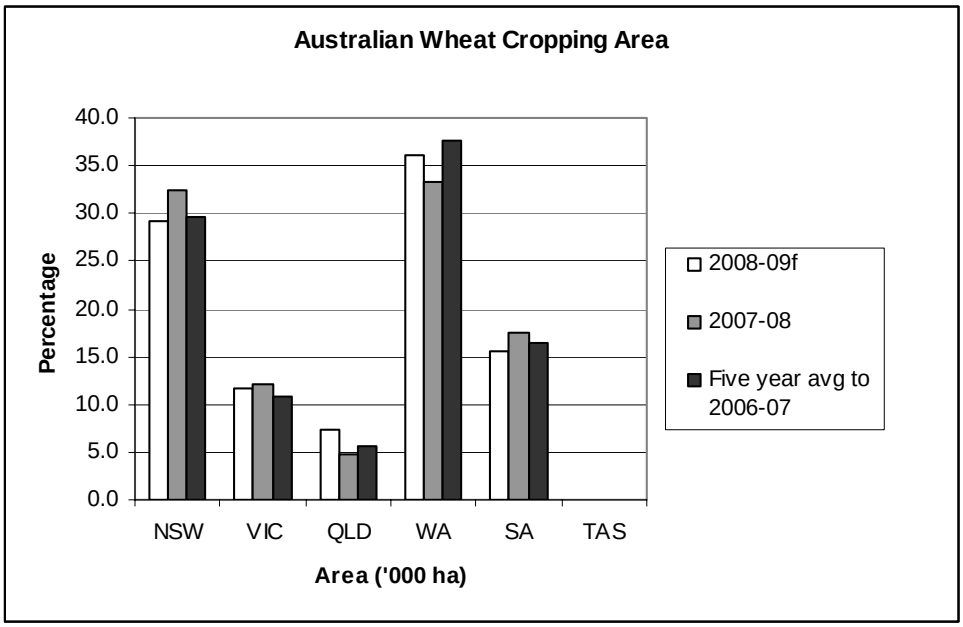


Figure 1-3: Area of wheat planted ('000ha) in the six states over the last five years (2002-2007), and the predicted season for 2008-09 *(Data source: ABARE 2008)*



For the cropping season of 2008-09 an increase in wheat production in Australia of approximately 72% to 22.5 million tons has been forecast, relative to the previous season (ABARE 2008). An increase in production also leads to increased exports. The expected tonnage to be exported will be nearly double that of 2007-08 (Table 1-2 and Table 1-3). Australia is a large exporter of wheat, attractive to many markets, especially those in the Middle East and South East Asia, where Australia holds a 16% share of the world's wheat trade (AWB 2008).

Table 1-2: Australian wheat production: comparing the past five years and the forecast from September 2008 on cropping area, yield and total production

	Five year average	2007-08	2008-09 forecast
Wheat			
Area Planted ('000ha)	12375	12345	13552
Yield (t/ha)	1.50	1.06	1.66
Production (kt)	18828	13039	22460

(Data source: ABARE 2008)

Table 1-3: Australian production, consumption and export of wheat over the last three cropping seasons

	2006-07 (kt)	2007-08 (kt)	2008-09 forecast (kt)
Wheat			
Production	25150	10822	22460
Domestic Use	6540	7381	6699
- Human and industrial	2287	2264	2287
- Feed	3672	4500	3740
- Seed	581	617	673
Exports	15969	8685	15689

(Data source: ABARE 2008)

Since the European settlement of Australia, wheat has been grown predominantly as a food source. The initial varieties used over 200 years ago were not adapted for Australia's hard environment and these first crops were low yielding. With the expansion of wheat production from coastal New South Wales into other states of

Australia, wheat production increased. This increase was also due to improved farming techniques, application of fertilizers and pesticides, the introduction of rotation crops and breeding of varieties specific to the Australian environment (AWB 2008).

In recent years breeding of wheat varieties has become essential in wheat production and is focused in particular areas of development, varying slightly for the different wheat producing areas of Australia. Breeding objectives are usually separated into sections, these being Yield, Biotic stresses, Abiotic stresses and Quality attributes. The GRDC (Grains Research and Development Corporation) is one of the major funding bodies for wheat breeding in Australia drawing on funding from Australian wheat producers and the Federal Government. Pre-breeding research objectives/priority traits are regularly determined by discussions between the GRDC, the Australian Winter Cereals Pre-breeding Alliance and breeding companies (Table 1-4). A more detailed look at wheat breeding objectives will be described in the following sections of this chapter.

Table 1-4: List of Priority traits/breeding objectives endorsed by the Pre-Breeding Alliance and the GRDC. *(Data source: GRDC 2008)*

Biotic Stresses	Abiotic Stresses	Quality
• Crown Rot • Leaf Rust • Root Lesion Nematode:(*Pratylenchus neglectus*) • *Septoria tritici* blotch • *Septoria nodorum* blotch • Stem Rust • Stripe Rust	• Drought tolerance • Water use efficiency • Frost tolerance • Salinity tolerance	• Defect elimination • Late maturity alpha-amylase • Pre-harvest sprouting • Dough mixing characteristics: (strength, extensibility, mixing time) • Flour water absorption • Milling yield • Quality for Asian noodles • Noodle manufacture • Bread making quality

1.1.2 *Wheat Production in China*

China's population is just over 1.3 billion people and represents 20% of the world's population of 6.7 billion. Therefore maintaining or increasing wheat production is important to China so that it can meet the needs of a growing population. China is the world's largest wheat producer, with a yield in 2007-08 of 109.9 million tons and has a predicted yield of 114 million tons for the 2008-09 season (Table 1-5). After a poor harvest in 2003/2004, the Chinese government has encouraged farmers to expand wheat production with incentive policies. USDA (2008a) states that these incentives include: tax reductions, direct subsidies for inputs and high quality seeds and minimum support prices.

Table 1-5: World Wheat Production and Consumption compared to Australia and China (units: thousand metric tons)

	2004/05	2005/06	2006/07	2007/08	2008/09 Oct
Production					
Australia:	21, 905	25,173	10,822	13,039	21,500
China	91, 950	97, 450	108,470	109,860	114, 000
World	625,738	620,851	596,304	610,883	680,200
Consumption					
China	102,000	101,500	102,000	104,000	107,000
World	606,681	624,182	616,927	618,102	655,585

(Data source: USDA 2008b)

The Chinese Academy of Agricultural Sciences has divided the country into ten zones of wheat production (Figure 1-4). These zones are determined by geographical location which then governs sowing and harvesting times. Zone V: is the South-western Autumn-sown spring wheat zone and includes most parts of the Sichuan Province. The Chinese varieties used in this study were developed and grown in Zone V.

Figure 1-4: Wheat production Zones in China. Separated by location and time of planting

Legend:

I Northern Winter Wheat Zone
II Yellow and Huai River Valleys Facultative Wheat Zone
III Middle and Low Yangtze Valleys Autumn-Sown Spring Wheat zone
IV Southern Autumn-Sown Spring Wheat Zone
V Southwestern Autumn-Sown Spring Wheat Zone
VI Northeastern Spring Wheat Zone
VII Nothern Spring Wheat Zone
VIII Northwestern Spring Wheat Zone
IX Qinghai-Tibetan Plateau Spring-Winter Wheat Zone
X Xinjiang Winter-Spring Wheat Zone

(source: Figure 1.3 Wheat zones of China. Chapter 1: "A history of Wheat Breeding in China" He et al. 2001)

Since 1949 wheat breeding in China has become more important and has proven very successful in producing varieties with higher yield potentials, a reduction in plant height which has aided lodging resistance and resistance to disease. For Zone V, the Sichuan Province, the major breeding objectives as stated by He *et al.* (2001) are to produce lines with a) high yield, short stature and with good lodging resistance; b) resistance to stripe rust, powdery mildew and head scab; c) early maturity to fit the multiple cropping system; c) drought tolerance for wheat sown in hilly areas without irrigation, and tolerance to poor soil fertility; and d) preferred white kernel colour.

Producing varieties with a high yield potential is essential to maintain the necessary increase in production. Establishing disease resistance in the breeding material is very important especially with rust and powdery mildew being such a large problem in China. Landraces have been used with lines from Italy, Mexico (CIMMYT), USA, Australia, Canada and other countries to improve the varieties grown by the local farmers (He *et al.* 2001).

The Ministry of Science and Technology of China (MOST 2006) has stated that between the years of 2006 – 2020 China aims to produce 50 new varieties of what they have termed "super wheat". The reports explain that these varieties will increase total wheat production by 30%. This equates to 33.3 million hectares with a forecasted output of 10,500 to 12,000 kg/ha and yield of 10.45 tons/ha (china.org.cn 2006).

1.2 Breeding Objectives in wheat improvement

It is the goal of a wheat breeder to create new genotypes with improved features compared to previous varieties (Poehlman and Sleper 1995). Parental material is selected to fulfil breeding objectives relative to the environment and economic market. A wheat breeder needs to consider the following when developing new genotypes: increasing yield potential and stability; improving tolerance to biotic and abiotic stresses; and maximising the end product quality.

1.2.1 *Yield Potential*

Yield of grain is of the primary importance to any cereal breeder as a high yield potential ensures appropriate economic return to the grower and the ultimate success of the variety. Yield potential and stability requires evaluation over several years and in a range of locations/environments before a new variety can be released. Traditionally, breeding for high-yielding potential is accomplished by the crossing of selected genotypes to supply genes for yield potential through the generation of transgressive segregates expressing a superior yield phenotype (Poehlman and Sleper 1995). Yield potential, a complex quantitative trait, takes into account a number of morphological and physiological features. It is a trait that may benefit with the use of modern molecular techniques when used with traditional breeding methods. It has been reported that there is a need to develop varieties that have high yielding potentials to ensure the rate of production equals or betters the rate of consumption. To maintain this, improved breeding methods based on physiological criteria have

been implemented (Reynolds 1998). This physiological selection was based on the genetic evaluation and mechanisms of stomatal aperture-related traits.

Fischer (2007) states that rate of yield progress (using conventional breeding methods) is between 0.5-1% per year and is limited by the following agronomic factors: available soil moisture, biological soil additives and seasonal climatic conditions. In the past yield has increased through the establishment of new varieties, improved farming techniques and the application of soil additives.

1.2.2 *Heat and Drought Tolerance*

Damage to wheat crops due to heat or drought stress is a common occurrence in the Australian environment, therefore breeding for heat and drought tolerance remains an important area for improvement. The effect of heat stress varies with the stage of plant growth and the duration of the stress, but is most critical during the flowering period, due to reduced pollen viability, stigma receptivity and seed formation (Poehlman and Sleper 1995). Genetic control of heat and drought tolerance is a combination of complex physiological and morphological traits and is therefore difficult to evaluate. Improved water use efficiency is a mechanism by which wheat varieties can support drought tolerance. An experiment of this type was carried out by Najafian *et al.* (2004) where varieties were planted under water stress at three locations to produce lines with high yield. McIntrye (2006) has been studying the genetic basis of obtaining drought tolerance in wheat, and is concentrating the search in genomic areas that code for flowering period, height, grain size and number and stem carbohydrates. As there are many traits that can contribute to a wheat plant

tolerating a high level of water stress, it continues to be a very complex area of research.

1.2.3 *Disease Resistance*

The development of wheat varieties with resistance to a range of disease pathogens is a major objective for wheat breeding worldwide. Each crop disease is treated as a separate breeding objective, as many have a high degree of pathogen race specialisation (Poehlman and Sleper 1995). Australian wheat breeding programs focus on a number of diseases, these include: Crown Rot, Root Lesion Nematode (*Pratylenchus neglectus*), Septoria diseases (*Septoria tritici* blotch, *Septoria nodorum* blotch) and rust diseases (stem rust, leaf rust and stripe rust) (GRDC 2008). In the Northern Region of Australia, breeding programs are developing resistant lines to: Yellow Spot, Common Root Rot, Barley yellow dwarf virus and Karnal Bunt (Smut) (QDPI&F 2008).

Breeding for disease resistant varieties can be a complicated process as the resistance may be controlled by a single or multiple genes and the disease pathogen can be active with a number of races. To take leaf rust as an example, a good source of partial resistance is contributed by the gene *Lr34*. To reduce the likelihood of this resistance gene being overcome by a change in the pathogen; durable adult plant resistance needs to be achieved. To undertake this, Singh and Rajaram (1992) suggest adding the Lr34 gene with two or three additional slow rusting genes.

There are other methods to prevent or control diseases during the cropping season, and these include: the use of partial resistant varieties, planting of disease free seed stock, eliminating contamination from weeds and crop rotation (QDPI&F 2008).

1.2.4 *Quality*

The market demands high quality products in all aspects of wheat production, from a sound wheat kernel, to high quality milled flour. Wheat quality is a complicated area of wheat breeding and Dewan (1988) states that quality can be broken down into four important factors: protein content, grain hardness, flour dough strength and milling quality (flour yield and flour colour). Milling procedures and end product requirements are affected by varietal type and the environmental growing conditions. Environmental growing conditions include the amount of rainfall, soil fertility and other stresses. In Australia there are three groups of wheat cultivars grown; hard or bread wheat; soft wheat and durum wheat. Flour milled from each of these groups differ significantly for a number of quality components, some of which are: flour yield, water absorption, particle size index and dough mixing time (Poehlman and Sleper 1995). Martin (1996) states that there have been measures put in place in breeding programs in the Northern regions of Australia to ensure that new varieties comply with the quality requirements of all Australian wheat. The quality requirements included bread wheats produced having a hard grain, high milling quality, medium dough strength and a high extensibility. This high level of grain and flour quality tailors Australian wheat for the domestic and export markets.

Within Australia a number of the large research institutes have concentrated research projects on wheat and flour quality, some of which include: the Value Added Wheat CRC, Molecular Plant Breeding CRC (WA), South Australian Research and Development Institute (SARDI) and Queensland Department of Primary Industries and Fisheries, along with collaborations with many Australian Universities. These programs all concentrate on the enhancement of quality, and are generating and

optimising analytical techniques that will help wheat breeders, researchers, growers, buyers and all processors of wheat in Australia.

1.2.4.1 *Noodle Quality*

There are a number of research programs established in Australia that concentrate on perfecting the quality of wheat grain for many types of Asian noodles. Given that China is a large importer of wheat from a number of countries, it is important to research the areas that influence noodle production, colour and taste. This will ensure Australia with a reliable source of high quality wheat for the growing export market into China (Brettell 2006).

There are a number of quality requirements to be considered with the production of Chinese noodles. He (1999) expresses the importance of protein type and quality in Chinese noodle production compared to other wheat end-products consumed in China. China has generated a set of quality standards for wheat flour used in these end-products (Table 1-6).

Table 1-6: The National standard of wheat flour specifications for various end-products consumed in China

	Ash (%)	Wet gluten (%)	Farinograph Stability (m)	Falling Number (s)
Bread	<0.60	>33.0	>10.0	250-350
Noodles	<0.55	>28.0	>4.0	>200
Dumplings	<0.55	28.0 - 32.0	>3.5	>200
Steamed Bread	<0.55	25.0 – 30.0	>3.0	250

(Data source: National standard for Wheat Flour, 1993 and He 1999)

Another important aspect of noodle quality is the texture and sensory perception of cooked noodles. A number of research groups have concentrated their studies in this area, and have used taste panels as a means of assessing the noodle quality (Hatcher *et al.* 2002, Storlie *et al.* 2006, Zhang *et al.* 2005). Storlie *et al.* (2006) established a taste panel to assess the influence the puroindoline genes and environment have on texture of fresh Chinese noodles. They reported that both the environmental growing conditions, and the puroindoline genes affecting grain hardness, had a significant effect on noodle softness and texture. Researchers Hatcher *et al.* (2002) established different requirements for suitable noodle palatability, illustrating that a fine flour particle size and minimal starch damage during milling combines to give a superior noodle after cooking. Other studies have continued using tests such as flour swelling indexes as a predictive tool of noodle quality during the production and cooking of the noodles (Hu *et al.* 2004, McCormick *et al.* 1991).

As early as 1970, Moss (1971) was researching the use of Australian wheats for noodle production, with the emphasis of the study directed at white Japanese noodles and yellow Chinese noodles. He found that noodle quality was affected in three ways: 1. noodle whiteness/brightness decreased with increased protein levels, 2. noodles had differing responses to boiling and absorption of water, 3. the intensity of the noodle yellowness was due to the alkaline medium used. The basic parameters for noodle quality have remained unchanged.

1.2.4.2 *Noodle Types*

A large proportion of Australian wheat exports enter eastern Asia, where its high quality makes it ideal for a number of end-products (Brettell 2006). These include

white salted noodles, yellow alkaline noodles, instant noodles, steamed bread and dumplings (Table 1-7). All of which are a staple part of the daily meals in many parts of Asia.

Table 1-7: Wheat products consumed in China *(Data source: He 1999)*

Food Type	**%**	**Noodle Variety**	**%**
Steamed bread including flat bread	46	Fresh noodle (home made)	33
Noodles and dumplings	39	Dry noodles (machine made)	24
Cookies and biscuits	6	Instant noodle (machine made)	21
Western bread	3	Fresh noodle (machine made available at store)	13
Others	6	Extended alkaline noodle (La Mian)	5
		Extruded noodle (Helu)	2
		Frozen noodle and others	2

The homemade fresh noodle is the most popular noodle in China, as they can be produced easily with basic equipment (He 1999).

White salted noodles are popular in Japan, South Korea and China. These noodles can be found in a number of forms, including fresh, dried, boiled and steamed. These noodles are required to have a smooth appearance and should be soft and elastic in the mouth therefore starch and protein quality are important aspects (Bushuk 1998, Crosbie *et al.* 1998). Yellow alkaline noodles (YAN) have the same mouth feel requirements as white salted noodles, but require high yellow flour colour that is stable. This yellow colour may then reduce the amounts of additives (alkaline salts) needed in the production of the YAN (Crosbie *et al.* 1998).

1.2.4.3 *Noodle Colour*

Colour is an important sensory aspect of Asian noodle production and maybe the most important as it is the first assessment made of noodles by a consumer (Bhattacharya *et al.* 1999, Crosbie *et al.* 1998, Mares and Panozzo 1999). Colour and discolouration of flour and dough products are affected by a number of quality factors. These being genotypic and environmental effects, protein content, colour of wheat pericarp, inherent pigmentations, flour processing conditions and storage conditions (Bhattacharya *et al.* 1999). Flour colour can also be affected by the milling of the wheat, whereby bran flakes/ash can enter the flour during the grain milling thereby hindering the whiteness of the flour (Brettell 2006). Flour colour can be affected by its inherent accumulation of carotenoids in the wheat grain endosperm. These yellow pigment components, usually of the Xanthophyll type, can produce a colour range from bright white to creamy yellow (Brettell 2006). Colour is not always constant; therefore colour stability of flour and end-products needs to be considered. A large amount of research has concentrated on the causal effects of colour and colour stability, and it is effect on the appearance of noodles, but it is the polyphenol oxidase (PPO) activity and Xanthophyll content that will be assessed in this chapter.

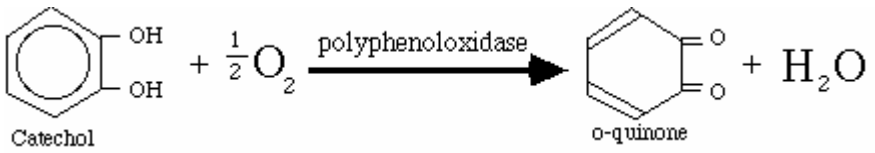
1.2.4.3.1 *Impacting factors on noodle colour i) Polyphenol Oxidase Activity Levels*

Discolouration of fresh noodles has been highlighted as a significant problem in the production of noodles in China. It has been shown that Polyphenol oxidase (PPO) activity is a cause of the time-dependent discolouration of fresh noodles

(Bhattacharya *et al.* 1999, Demeke *et al.* 2001, Kruger *et al.* 1992, Mares and Panozzo 1999). It has been reported that PPO can be found on the seed coat of wheat grains and it is an enzyme which catalyses the oxidation of phenolic acids to quinones. These react with amines and thiol groups or undergo self-polymerisation to produce dark brown or black pigments (Demeke *et al.* 2001, Steffens *et al.* 1994) (Figure 1-5).

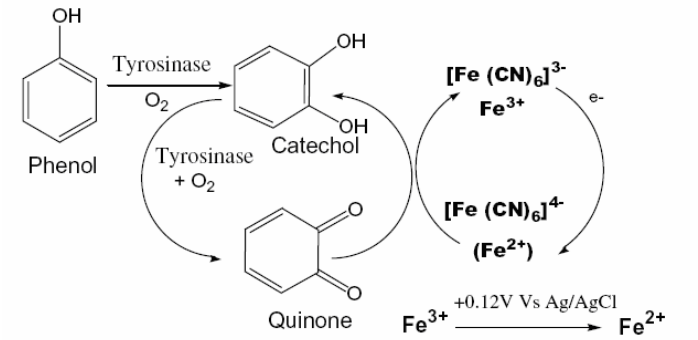
Figure 1-5: The reaction catalysed by Polyphenol oxidase *(a: Data source: http://plantphys.info/plant_physiology/enzymelab.html) (b: Data source: Rajesh et al. 2005)*

a)



(di-hydroxyphenol)

b)

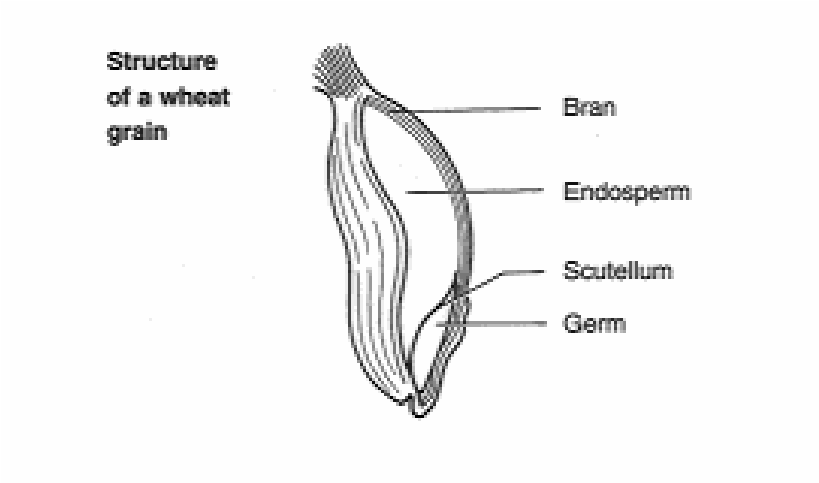


Many studies have been undertaken to both understand the mechanism by which this enzyme works, and to measure the PPO activity of harvested grain products.

Kruger (1976) has found evidence for the presence of 12 isozymes of PPO in different parts of the wheat kernel. This study also found that as the kernel approached maturity, the level of PPO in the endosperm dropped and the PPO in the germ and scutellum increased (Figure 1-6). These findings are consistent with the research of

Taneja *et al.* (1974) who also reported that the number and type of isozymes decline once the seed is fully developed.

Figure 1-6: Diagram of the wheat grain *(Data source: http://www.nutrition.org.uk)*



Demeke and Morris (2002) has studied the PPO gene and characterised the gene in wheat. This research has produced a significant polymorphism between DNA fragments amplified from different cultivars. This polymorphism can clearly separate cultivars with a high PPO from those with a low PPO activity. The Polymerase Chain Reaction (PCR) was performed by these researchers using genomic DNA and oligonucleotides designed from the conserved binding regions in all PPO genes. It is the conserved copper binding regions that are responsible for the interaction with oxygen and phenolic compounds (Steffens *et al.* 1994). Demeke and Morris (2002) were successful in amplifying a DNA fragment which showed a high level of sequence homology to other plant PPO genes in a BLAST search.

Research has also taken place to determine the location of the PPO genes on the wheat genome. Mares and Campbell (2001) identified Quantitative Trait Loci (QTL)

associated with PPO activity on chromosome 2A and 2D using a doubled haploid population. Demeke *et al.* (2001) identified QTLs located on chromosomes 2A, 2B, 3D and 6B in a recombinant inbred line population. This research group found different QTLs were identified using different PPO substrates, L-DOPA and L-tyrosine, whereas Mares and Campbell (2001) only employed L-tyrosine to generate their phenotypic data.

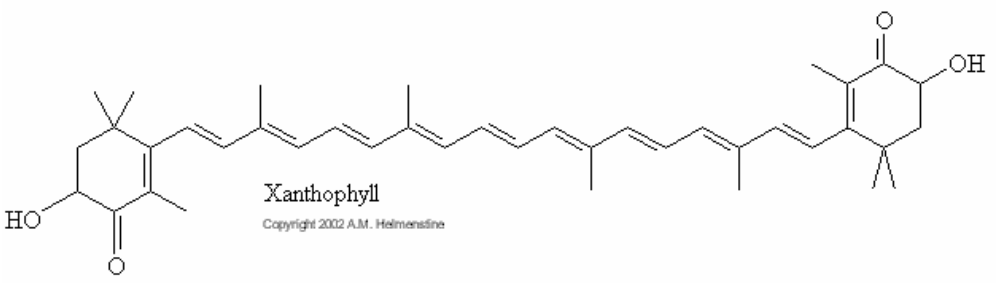
Raman et al (2005) have also identified a significant QTL on 2A for PPO activity in a doubled haploid population, again using both PPO substrates. In order to develop functional gene markers Raman *et al.* (2007) have developed a SNP and a CAPS marker tightly linked to the QTL identified on chromosome 2A.

In the past, selection on wheat germplasm for varying PPO activities has been conducted through the development of whole seed assays using particular PPO substrates to generate high quality phenotypic data for line selection and QTL identification research (Bernier and Howes 1994, Demeke *et al.* 2001, Demeke and Morris 2002, Mares and Campbell 2001, Raman *et al.* 2005, Raman *et al.* 2007).

1.2.4.3.2 *Impacting factors on noodle colour ii) Xanthophyll content*

Plant tissue contains pigments that contribute to the colour of that tissue. In wheat flour, the pigment that constitutes part of its yellow colour is the carotenoid pigment, xanthophyll (Moros *et al.* 2002). Xanthophyll is a complex structure of alternating double and single bonds and it is this structure that contributes to the compounds shape, chemical reactivity and light absorbing properties (Figure 1-7).

Figure 1-7: Chemical structure of Xanthophyll *(Data source: http://chemistry.about.com/library/graphics/blxanth.htm)*



The light absorbing properties of xanthophyll produces various shades of red to yellow colours in foods (Moros *et al.* 2002). Lepage and Sims (1968) developed a method to characterise the carotenoid pigments in wheat flour, demonstrating that they are composed of xanthophylls in the form of free lutein and its esters. Total xanthophylls can be extracted from wheat flour using water-saturated butanol or methanol (Burdick 1956). This method can be used as a predictive tool to assist in the selection of wheat flours for the Asian noodle market since it is now recognised that the variation in noodle 'yellowness' is due to the presence of xanthophylls (Mares *et al.* 1997). It has been shown that xanthophyll content has an impact on the stability of colour in fresh noodle sheets. Mares et al (2000) reported a decrease in brightness and whiteness in fresh noodles over time. This was associated with a 20 - 37% decrease in xanthophyll content in the first 6 hours of noodle production. After this time the noodle colour and the xanthophyll content remained more constant. This study also indicated that the genotypes that contained the most xanthophyll had relatively low rates of darkening.

Miskelly (1984) first detected a correlation between flour colour and Asian noodle sheet colour. Subsequent research by Elouafi *et al.* (2001); Mares and Campbell

(2001) and Parker *et al.* (1998) have identified chromosomal locations of linkages between yellow pigment in wheat to chromosome 7. Parker *et al.* (1998) identified QTL's for flour colour on 3B and 7A in a 'Schomburgk x 'Yaralinka' single seed descent population accounting for 13% and 60% of the genetic variation respectively.

1.3 Breeding methods used in wheat improvement

There are a number of breeding methods that can be used to select agronomically superior lines from populations containing desired traits. The lines become varieties only after they are selected from segregating populations and are carried through until homozygosity is reached following at least one hybridisation (Stoskopf *et al.* 1993).

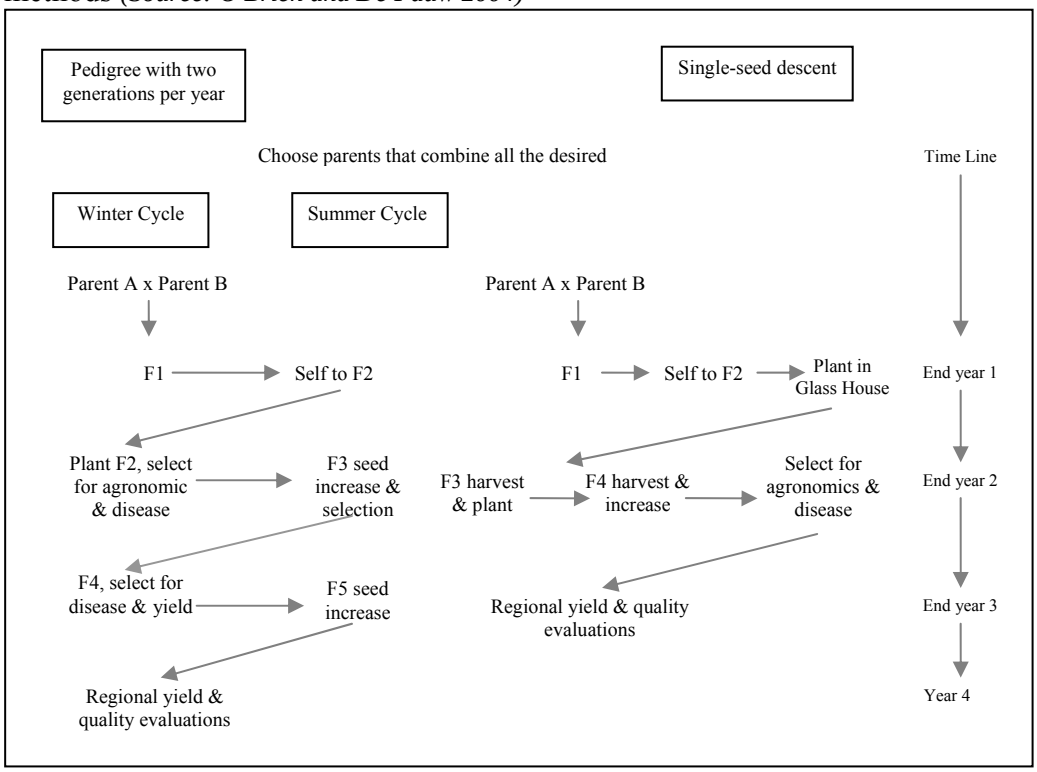
1.3.1 *Pedigree Breeding Method*

The pedigree system is a widely used method which produces new superior varieties and pure lines for breeding programs (Moreno-González and Cubero 1993). This method is used to introduce disease resistances and simply inherited traits into new varieties (O'Brien and De Pauw 2004). The F_2 generation, produced by crossing two lines both contributing beneficial alleles, is the first opportunity for selection (Figure 1-8). Plants are then re-selected in each subsequent generation until a level of homozygosity is reached and the plants express the desired phenotype (Poehlman and Sleper 1995, Stoskopf *et al.* 1993). Pedigree breeding is a simple and effective method, but is also labour intensive as extensive field trials are required (Moreno-González and Cubero 1993, Stoskopf *et al.* 1993).

1.3.2 *Single Seed Descent*

The Single Seed Descent method has been used in breeding programs when large numbers of lines need to be advanced in a short period of time. Only one seed is required from each plant each generation and this permits progression to homozygosity in limited spaces (Figure 1-8). Controlled environmental conditions in growth chambers and glasshouses allow more than one generation to progress a year thereby shortening line development time (Moreno-González and Cubero 1993, Poehlman and Sleper 1995). This method reduces the loss of genotypes with desirable alleles during the segregating generations, by maintaining a large number of F2 plants and advancing them through another four to six generations (O'Brien and De Pauw 2004). Other than ensuring speed and simplicity, another advantage of the Single Seed Descent method is that it is suitable for traits that have low heritabilities and where visual phenotyping is difficult (Moreno-González and Cubero 1993).

Figure 1-8: Schematic Diagram of the Pedigree and Single-seed decent breeding methods *(Source: O'Brien and De Pauw 2004)*



1.3.3 *Recurrent Selection Method*

Recurrent selection is a method that combines the techniques of both single seed descent and pedigree breeding to improve adapted genotypes in breeding programs (Stoskopf *et al.* 1993). "Recurrent selection is a population improvement method that increases the frequency of favourable alleles while maintaining genetic variation in breeding programs" (Doerksen *et al*. 2003). To accomplish this increased frequency of desirable alleles, superior genotypes are selected and intercrossed to produce the next generation (Poehlman and Sleper 1995). The technique requires artificial emasculation in self-fertilising plants, therefore being more easily used in cross-fertilising plants (Moreno-González and Cubero 1993).

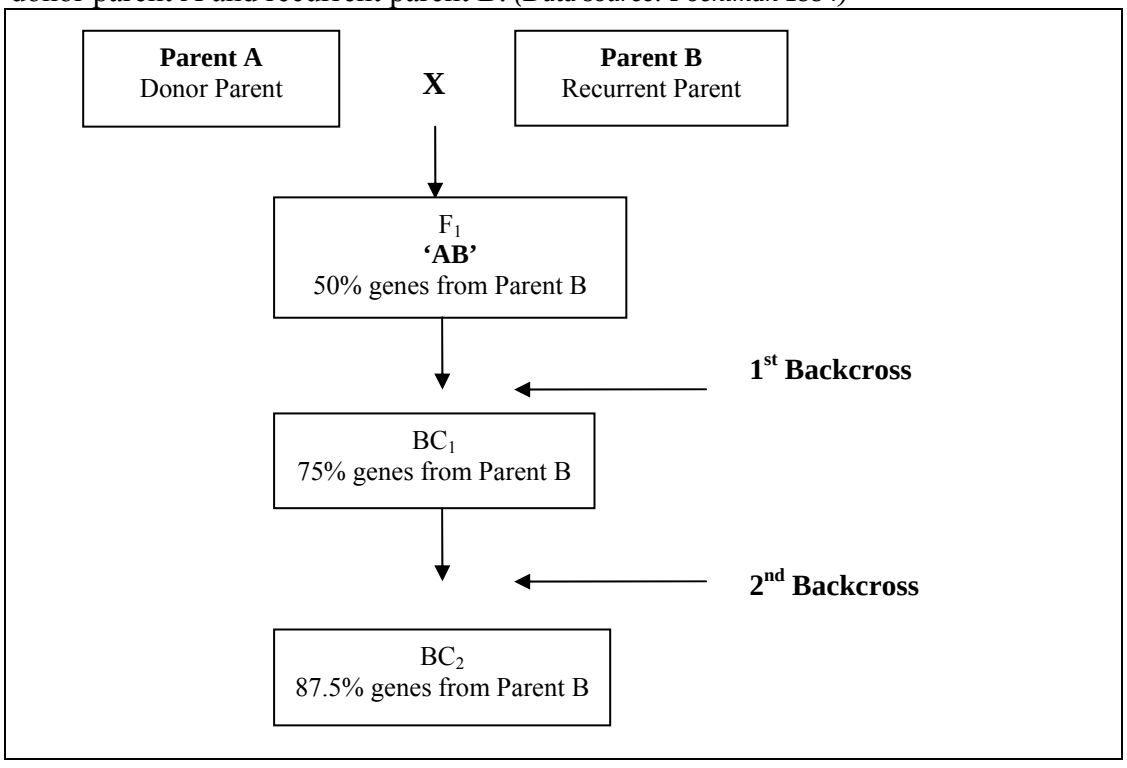
1.3.4 *Backcross Breeding*

Backcrossing is a technique that allows important genes to be transferred from one genotype into another with relative ease. Backcrossing is a form of recurrent hybridisation which substitutes a desirable allele for a particular trait with the current allele in an adapted cultivar. The method is carried out with an initial cross between a donor and a recurrent parent. The progeny produced in the F_1 generation is then crossed with the recurrent parent. The number of backcrosses required is dependent on the performance of the donor alleles within the recurrent parent genotype and the proportion of homozygosity needed (Moreno-González and Cubero 1993). After the first backcross to the recurrent parent, the progeny have 50% of the alleles from the recurrent parent. After only three more backcrosses to this recurrent parent the progeny will have 93.75% of the alleles from the recurrent parent (Figure 1-9). The types of genes that can be considered for this technique are: single dominant genes,

single recessive genes and polygenes. The method can become more complicated when the trait is more complex (Poehlman and Sleper 1995). When considering several genes, they can be transferred simultaneously or in a stepwise progression. To introgress two or more genes stepwise, each gene is backcrossed into the recurrent parent separately and then assessed at the end. Simultaneous selection of several genes is a more complex method and requires large plant populations.

Stoskopf *et al.* (1993) explains for a backcross population to be successful, the following is required; an ability to identify the trait being transferred; a simply inherited trait; and a sufficient number of backcrosses carried out to recover the recurrent parent genotype.

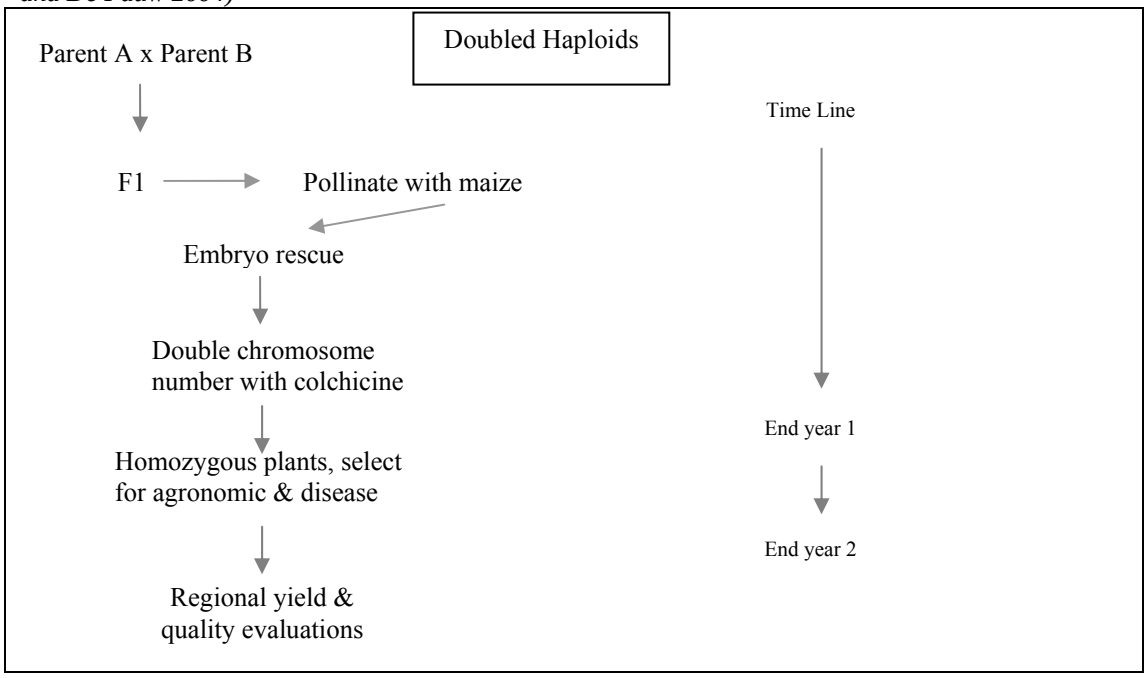
Figure 1-9: Backcrossing Technique used in self pollinating plants. A cross between donor parent A and recurrent parent B. *(Data source: Poehlman 1994)*



1.3.5 *Doubled Haploid Populations*

The development of doubled haploid populations has become important in wheat breeding in recent years because of their usefulness in molecular marker development and identification for desirable traits. The major benefit of doubled haploids is that homozygosity is achieved in a very short time compared to other breeding methods (Stoskopf *et al.* 1993). To produce these populations, a number of steps are required. Firstly, haploid plants are generated through crossing of wheat spike florets with collected maize pollen. The maize chromosomes are lost and haploid wheat embryos form which will abort if left in the developing grain. Consequently the forming haploid embryos are "rescued" to plant growth media and incubated under a sequence of conditions which stimulate embryo germination and plantlet formation. These small sterile plants are then transplanted and grown in soil. At the tillering stage they are treated with colchicine which artificially doubles the chromosomes in some cells within the crown, generating diploid tillers that produce fertile seed (Figure 1-10) (Kammholz *et al*. 1996, Kammholz (2001) *PhD)*. These fertile seed are homozygous at all alleles, and therefore beneficial for research purposes and for the release of varieties in a shorter time period (O'Brien and De Pauw 2004).

Figure 1-10: Schematic Diagram of production of Doubled haploids, *(Source: O'Brien and De Pauw 2004)*



1.4 Genetic markers for wheat improvement

1.4.1 *Types of Markers*

A genetic marker is a score-able identifier linked to a trait of interest at particular chromosomal location.

There are three broad classes of markers, these being morphological, biochemical and molecular. Morphological markers have been associated with traits that can be visually assessed, but these types of markers have limited success in wheat. Secondly biochemical markers have been used within wheat breeding, but their use is limited by the lack of functional biochemical markers available. Biochemical markers can be identified by assays for the production of particular proteins. For example

glutenin proteins can be extracted and visualised by electrophoresis (Eagles *et al.* 2001). Because of the limitations of the above marker types in recent years there has been a move towards using molecular markers. There are a number of different types of molecular markers frequently used in wheat research. These being; random amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs or microsatellites) and single nucleotide polymorphism (SNPs). RAPDs and RFLPs were frequently used in the early years of molecular research into wheat but were replaced by SSRs and SNPs because of their low levels of polymorphisms and difficultly in use. AFLPs have been used less in recent years as they restrict researchers to only dominant markers (Landjeva *et al.* 2007). Overall SSR markers have been a very popular marker type in wheat genetic studies as they are highly polymorphic, reproducible and are co-dominant in nature (Röder *et al.* 1995). As breeding programs are demanding large number of lines genotyped by a number of different markers, SNPs are becoming more important (Landjeva *et al.* 2007). Varshney *et al.* (2005) states that expressed sequence tag (EST)-derived SNPs and SSRs are highly functional molecular markers as they have been developed from gene sequence data. These markers have an advantage over other marker types because they are linked to the desired trait allele. This is an important step in generating true associations between markers and QTLs (Quantitative Trait Loci), which is linked to a particular gene of interest. The closer the marker is to being perfect (the gene), the more useful they are to breeders.

The usefulness of any molecular marker relies upon its ability to reveal polymorphisms within important breeding material and to make breeding programs more efficient than those currently in use.

1.4.2 Applications of markers in wheat breeding

Both Babu *et al.* (2004) and Francia *et al.* (2005) have reviewed the number of factors/requirements that influence the success of marker application in breeding programs, and these are: a) a genetic map with an adequate number of uniformly spaced markers for the location of desired QTL/s of major genes; b) a tight association of desired QTL/s or major gene of interest and flanking markers ; c) an adequate recombination between the markers and the rest of the genome; d) an ability to analyse a number of plants in a time and cost effective manner. Young (1999) states that scientists can maximise the efficiency of DNA markers by using them to introduce novel traits of interest into breeding programs, rather than using them to just perform tasks of conventional breeding.

Before molecular markers can become a mainstream asset to breeding programs, their individual usefulness has to be demonstrated. It is very important to establish a link between the markers identified and the QTLs/traits they are associated with. To do this marker validation has to be carried out for all traits, over a number of environments and in multiple genetic backgrounds to test the robustness of these markers.

Christopher and Banks (2002) explains the necessary steps for marker validation as: "1) markers must be assessed for technical robustness and transferability; 2) markers must be assessed for polymorphism; 3) markers must be assessed in a cross involving local breeding material to establish that a similarly close linkage exists as has been identified in the original mapping population."

There has been a large input of funding for the development and identification of molecular markers useful to plant breeders. The next step is for these markers to be used as a tool to predict phenotype and enhance the development of breeding lines towards new varieties. William *et al.* (2007) describes the ways that breeding programs can benefit from the introduction of molecular markers: a) to pyramid genes for specific traits; b) to select for recessive genes; c) to use when phenotypic screening is difficult; d) to use when the environment can affect the phenotype; e) to be used when the heritability of a particular trait is low and f) can be used at early growth stages; with seed or young plant tissue.

There are a number of molecular markers that have been used on a regular basis in wheat breeding programs across Australia to assist the introduction of specific traits into elite breeding material (Table 1-8).

Table 1-8: Markers that are currently used in wheat breeding programs across Australia

Traits assessed with markers used Australia wide	Marker
Cereal Cyst Nematode resistance	*Cre1; Cre3*
Pratylenchus neglectus resistance	*Rlnn1*
Noodle Quality (Granule bound starch synthase)	*Wx-A;Wx-B1;Wx-D1*
Boron Tolerance	*Bo1; Bo2*
Stem Rust resistance	*Sr2; Sr39; Sr36; Sr24/Lr24*
Grain Protein	*Nor1*
Coleoptile length	*Rht8*
Tiller inhibition	*Tin*
Barley yellow dwarf virus	*Bdv2*
VPM rust resistance	*Sr38/Lr37/Yr17*
Flour colour	*FC3*

(Data source: Eagles et al. 2001)

Christopher and Banks (2002) has reported that markers linked to traits important to the Northern wheat region of Australia are also being integrated into the breeding programs. These traits being: rust resistances, russian wheat aphid resistance, late maturity alpha-amylase activity (absence of), polyphenol oxidase activity, grain hardness, semi-dwarfing status, crown rot resistance, root lesion nematode resistance (*P.thornei*), yellow spot resistance, pre-harvest sprouting resistance, black point resistance, max extensibility, flour milling yield and flour water absorption.

1.4.3 Construction of genetic linkage maps

As a very large genome of $16x10^9$ base pairs, undertaking genome wide studies on wheat is a difficult and time consuming effort. A number of studies have been successful in developing large numbers of microsatellite (SSR) markers with the purpose of generating wheat linkage maps. The Wheat Microsatellite Consortium (WMC) is an international collaboration with the main objective of developing microsatellite markers to be mapped on the International Triticeae Mapping Initiative (ITMI) map (W7984 x Opata85). This mapping population has been used to map 396 SSRs which were developed through WMC, 279 SSRs developed by Röder *et al.* (1998), 55 SSRs by Pestsova *et al.* (2000), 66 SSRs by Gupta *et al.* (2002), 53 by Stephenson *et al.* (1998), 337 SSRs by Sourdille *et al.* (2001), 144 SSRs by Harker *et al.* (2001) and 347 SSRs by Song *et al.* (2005).

The process of generating a genetic linkage map is made possible by the above numbers of microsatellite markers available. Three major steps are required to construct a linkage map; 1) Development of a mapping population; 2) Screening of

the markers against the parents of the population; 3) Generating genotypic data from the population for each polymorphic marker.

There are a number of population types that can be used for mapping, these being, doubled haploids, Recombinant inbred lines, backcross populations and F_2 populations (Langridge and Chalmers 2005). The mapping population is dependent on the phenotype or specific traits that are being investigated, and it is the selection of parents that holds the most importance in this step (Grandillo and Fulton 1998). The size of the population has a large affect on the linkage map, and its ability to generate the correct marker order. Mapping populations can vary in size, as in the doubled-haploid populations generated by Kammholz *et al.* (2001), where the five populations produced contained numbers up to 321 lines. Wu *et al.* (2003) states that the ordering power of markers in mapping populations depends on the population size and the marker distances. If the marker distance is to be 5cM apart, then the mapping population needs to consist of 200 lines. If the marker distance is increased to 10cM then the required population size is a more manageable 150 lines.

Mapping populations generated by wide crosses will generate the high level of polymorphism required in the marker screening step. The polymorphic markers are then assayed across the entire population to generate the genotypic data required to produce a linkage map. Computer programs, most of which are freely available over the internet are used to perform the linkage analysis. Some of these programs include: MapMaker/EXP (Lander *et al.* 1987); JoinMap (Stam 1993); MapManager QTX (Manly *et al.* 2001); RECORD (Van Os *et al.* 2005) and Q-gene (Nelson 1997).

The analysis required to link markers together is based on the maximum likelihood ratio (LOD) and it is a LOD score value of >3 that is generally considered to indicate a significant linkage between markers (Collard *et al.* 2005). It is suggested by Hackett and Broadfoot (2003) that the effects of scoring errors reduce the accuracy of maps impacting the length of the linkage map as well as the marker order. Therefore managing linkage maps as indicated by Lehmensiek *et al.* (2005) will ensure that genotypic data collected will not hinder any further analysis.

1.5 Quantitative Trait Loci (QTL) analysis

1.5.1 Methods of detecting QTLs

QTL analysis is carried out to identify a linkage or association between a particular phenotype and the genotype of markers (Collard *et al.* 2005). The three methods for detecting QTLs used on a regular basis are single-marker analysis, simple interval analysis and composite interval analysis.

Mapping software like Map Manager QTX allows all three of these methods to be carried out. Single-marker analysis is a linear regression that tests for an association between the phenotypic values with the genotypic data of a single marker locus (Manly *et al.* 2001). The linear regression produces a R^2 value which explains the amount of phenotypic variation being explained by that particular marker. Another method, Interval Mapping can be carried out to identify a QTL and to estimate its position on the genome. Interval mapping analyses two markers at a time that are located next to each other on the same chromosome (Lander and Botstein 1989). The

next step on is Composite Interval Analysis which Manly *et al.* (2001) explains as a method of using the most significant QTL to control the effects of subsequent significant QTLs. This method was developed by Zeng (1994) to undertake QTL analysis and identification by taking into account the effects of possible multiple or linked genes.

1.5.2 Factors affecting QTL analysis

Reliable identification of QTLs is very important if these QTLs are to be used in Marker assisted selection. The accuracy of QTL analysis can be hindered by a number of factors, one of which is the reliability of the related phenotypic data. To detect QTLs with small effects, having conclusive phenotypic data is essential (Collard *et al.* 2005). Asins (2002) has reviewed the use of QTLs in plant breeding and has highlighted other factors that can affect QTL analysis. These are: the marker order of the linkage map, environmental variation, and epistatic effects from other QTLs. Accurate mapping and linkage map generation can be supported by an appropriate experimental design, population size and robust molecular markers. When the environment and other QTLs/genes are influencing the phenotypic and genotypic data advanced methods of QTL analysis including Multiple QTL Mapping (Jansen 1993) and Composite Interval Mapping (Zeng 1994) can assist in improving QTL identification.

Young (1999) emphasises that for high quality QTL analysis and identification of robust DNA markers, the following needs to be maintained: a) high quality scoring

methods; b) large population sizes; c) multiple replications and environments and d) validation of markers.

1.6 Marker Assisted Selection (MAS)

Through the aid of molecular markers, Marker Assisted Selection (MAS) is a technique that selects plants with particular genes/QTL involved in the expression of traits of interest (Babu *et al.* 2004). With the development of molecular technologies, it is now possible to combine molecular markers with phenotypic data collected from the field, to increase the efficiency of selecting superior lines. A modified method of using markers in the early generations and combining the phenotypic selection in the later generations has been recommended as an efficient system to integrate MAS into breeding programs (Liu *et al.* 2004). MAS has been indicated to be a useful technique at the times when conventional phenotyping procedures are too expensive, time consuming or unreliable (Francia *et al.* 2005, Koebner and Summers 2003).

MAS in plant breeding has the ability to save time by selection of genotypes at early stages of plant development. This method also assists the minimisation of linkage drag, selection for traits with low heritability, the introgression of specific traits of interest and assists gene pyramiding into elite varieties (Collard *et al.* 2005, Francia *et al.* 2005, Hospital and Charcosset 1997). There have been a number of simulation studies performed to assess the effectiveness of MAS as a breeding tool. The main conclusions from these studies are that in large populations, when heritability values are low and the markers are relatively close to the QTLs, MAS could be more efficient than pure phenotypic selection (Edwards and Page 1994, Hospital *et al.* 1997, Lande and Thompson 1990, Moreau *et al.* 2004, Moreau *et al.* 1998). It has

been shown that using molecular markers at early stage generations can be a very efficient use of MAS, as the rate of fixation of QTLs with larger effects is faster with markers compared to phenotypic selection (Koebner and Summers 2003, Kuchel *et al.* 2007). Also applying MAS at early generations reduces the complication of recombination between molecular markers and the trait of interest/QTL (Edwards and Page 1994, Hospital *et al.* 1997, Ribaut and Betrán 1999).

1.6.1 Gene/QTL Introgression

In order to use MAS successfully great care is needed to choose the most appropriate marker. Having a molecular marker located within the gene being transferred is the most favourable strategy for MAS (Babu *et al.* 2004, Dubcovsky 2004, Francia *et al.* 2005). It is generally difficult to find these ideally located molecular markers especially when considering polygenic traits. In these instances a genomic region carrying the QTL or genes is transferred using two flanking markers. It is important that the distance between the flanking markers is no larger than 20cM. The chance of recombination events occurring lessens when the markers are close to the gene (Babu *et al.* 2004, Edwards and Page 1994, Francia *et al.* 2005, Moreau *et al.* 1998). It is agreed that tightly linked markers is a key element of MAS having an advantage over pure phenotypic selection. This advantage cannot be maintained as the distance between marker and QTL increases (Chao and Ukai 2000, Edwards and Page 1994). Francia *et al.* (2005) states that there are limitations to using QTL mapping information in breeding through the use of MAS. These limitations include: accurate identification of the QTLs controlling the trait; uncertainty of the QTL position; incorrect measurement of the QTLs effect; limitations of QTL-marker associations

over a range of breeding material; the possibility of loosing the target QTL through double crossover events during MAS; and the difficulty in assessing other limiting effects. Manipulating complex polygenic traits through MAS is a more complicated effort. When several genes are involved the plant tends to exhibit smaller phenotypic effects, therefore making it more difficult to capture using conventional methods as well as using MAS (Ribaut and Hoisington 1998). There have been successful studies completed by a number of researchers worldwide. The first studies into this area were those that simulated MAS through computer programs in different plant species to assess the possibility of introgressing one or more genes/QTLs into elite breeding material (Frisch and Melchinger 2001, Knapp 1998, Ribaut *et al.* 2002, Reyes-Valdés 2000). In recent years there have been a number of studies that have introgressed genomic regions from wild relative germplasm (Fulton *et al.* 1997, Robert *et al.* 2001); and QTLs (Toojinda *et al.* 1998, Tomar and Menon 1998) into useful and agronomically successful varieties in tomato, barley, wheat and other plant species.

1.6.2 Marker Assisted Backcrossing

Marker-assisted Backcrossing has been a means of transferring genomic regions from a donor parent (variety or wild relative germplasm) into elite breeding material. Marker assisted selection can be very useful backcross breeding when it is required to select the lines with the necessary amount of target genomic material but an otherwise high proportion of recurrent parent germplasm. This reduces the amount of 'linkage drag' occurring. Markers can also assist in selecting the lines that have undergone recombination between the recipient DNA and the target gene/QTL introgressed (Babu *et al.* 2004, Francia *et al.* 2005).

Under conventional backcross breeding methods, recovery of all the recurrent parental genome would typically take 6-8 backcross generations (Allard 1960, Collard *et al.* 2005). With the aid of tightly-linked flanking markers conferring a trait of interest and background (recipient DNA) marker selection, the efficiency of recurrent parent recovery is greater and can be reduced down to three generations (Arús and Moreno-González 1993, Frisch *et al.* 1999, Frisch and Melchinger 2001). Therefore with a combination of backcrossing techniques and molecular markers, a large step ahead can be made within wheat breeding programs.

Researchers have successfully put the theory of MAS into practice in a number of plant species. Through the use of marker-assisted backcrossing, Steele *et al.* (2006) was able to successfully introgress five chromosomal segments into a rice variety. To complete such a feat, this research undertook many steps to complete their task; initial mapping, three backcrosses, two further crosses, thousands of marker assays, phenotyping and validation. All of which took eight generations and six years to produce one ideotype that contained all the five target regions in an elite rice variety. Kuchel *et al.* (2007) also had success in transferring disease resistant traits into an important wheat line. They achieved this with a shorter period of time than other researchers because they introduced a doubled haploid step into their methods. This technique may not be an ideal step to use in all breeding programs as it does increase the cost. In a similar manner, another successful example of MAS was reported by Toojinda *et al.* (1998) whereby a disease resistance QTL introgressed into a barley line.

Advanced backcross QTL analysis (AB-QTL) is a technique that was proposed by Tanksley and Nelson (Tanksley and Nelson 1996, Tanksley *et al.* 1996) as a mechanism to transfer QTLs of important traits into a variety using wild species germplasm. Other studies have been carried out whereby genomic regions and QTLs have been transferred through marker assisted backcrossing to enhance breeding lines or elite material (Huang *et al.* 2003, Huang *et al.* 2004, Tomar and Menon 1998, Yousef and Juvik 2002). Varshney *et al.* (2005) states that large populations sizes are required for selection of the useful alleles and maybe a limitation of AB-QTL.

1.6.3 Incorporating MAS into breeding programs

Markers have been used to characterise parental material used within breeding programs, allowing this information to improve the efficiency and effectiveness of selection in crosses and in segregating lines throughout the selection process (William *et al.* 2007). The integration of molecular marker technologies into conventional breeding strategies could produce genetic gains with greater speed and efficiency (Babu *et al.* 2004). Babu *et al.* (2004) also suggests using molecular markers in breeding strategies at one key selection step can maximise their impact as a prediction or diagnostic tool. It provides a reliable method of selection without the effect of environmental conditions. It is a method that is becoming more attractive with wheat breeders and is being used for traits that are difficult to screen for through traditional phenotyping methods (O'Brien and De Pauw 2004).

Francia *et al.* (2005) states that one of the most important aspects to ensure MAS efficiency in large breeding programs is the implementation of high-throughput molecular marker techniques that are efficient in both time and cost. Also, obtaining

the most effective marker for the trait of interest is imperative. New technologies into comparative genetics may identify positions of genes associated with the QTLs previously discovered. Having more closely linked markers will then improve MAS for complex traits.

A successful integration of MAS into wheat breeding programs has occurred in Australia with the introduction of resistance to cereal cyst nematode into wheat lines (Eagles *et al.* 2001). The two *Cre* genes have assisted the protection of wheat varieties from a disease that can significantly reduce wheat production in southern Australia. Testing for cereal cyst nematode is a high cost, unreliable bioassay, therefore this pest was an ideal test case for MAS.

Dubcovsky (2004) reports that MAS programs are a positive way to link wheat genomics with wheat breeding. It is important that the transfer of valuable genes into selected breeding programs continue therefore improving wheat varieties available for commercial growers. A combined effort between wheat researchers and breeders has shown to be successful in Australia through the National Wheat Molecular Marker Program implemented in 1996. Likewise in the United States of America, with the program MASwheat, which has allowed well documented genomic techniques to be easily accessed so that they can be used to implement MAS within wheat breeding programs worldwide (Dubcovsky 2004).

Computer simulations have been used to investigate the effectiveness of using markers to increase genetic gain and have found combining MAS with phenotypic evaluation a useful combination (Lande and Thompson 1990, Hospital *et al.* 1997).

Hospital *et al.* (1997) has found alternating MAS with and without phenotypic selection over several successive generations to be more efficient that using purely phenotypic selection, as well as being more cost effective. Integrating phenotypic assessment with MAS allows the markers to be re-evaluated at each generation. Without re-evaluating the markers, the additional gain provided by MAS rapidly decreases with several cycles of selection due to segregation of marker and trait (Hospital *et al.* 1997).

Ribaut and Hoisington (1998) state that using MAS to incorporate single-gene traits of interest has proved successful, however when considering polygenic traits, more innovative strategies need to be developed. To date MAS has been used in breeding programs to transfer genes or QTLs of polygenic traits when the phenotypic testing for these traits is difficult. Holland (2004) states that implementation of MAS for complex traits is dependent on accurately identifying, locating and estimating the effects of the QTLs. These effects also need to be seen over a number of important breeding lines to be a useful tool in plant breeding.

1.7 Rationale for the Current Study

A comprehensive study was carried out by Mares and Campbell (2001) whereby characteristics of flour and noodle colour were investigated in crosses of Australian wheat varieties. Major parameters linked to flour and noodle colour were found to be: Flour protein levels, Flour colour L* (brightness) and b* (yellowness), PPO activity and Xanthophyll content. The discolouration of fresh White Salted noodle and Yellow alkaline noodle sheets was investigated over defined periods of time,

measuring brightness and yellowness of both noodle types. QTLs were identified in three doubled haploid populations, one population being the Sunco x Tasman population. In this population, a highly significant QTL was identified on chromosome 2D for PPO activity, located in the same area contributing to the time dependent darkening and brightness of noodles, and to a lesser degree on a QTL located on chromosome 2A. QTLs significantly affecting Xanthophyll content were also identified in the Sunco x Tasman population, located on chromosomes 3B and 7A. These QTLs are located within regions corresponding to b* (yellowness) levels within flour and noodle sheets.

This evidence for links between PPO activity and noodle colour stability and between Xanthophyll content and noodle colour provided an excellent starting place for the current project.

When the current project commenced research into the practical aspects of Marker-assisted selection was limited. The majority of the research into this area were computer simulation studies concentrating on the comparison between the traditional phenotypic methods and the marker-assisted methods, using the backcrossing breeding method (Kruger *et al.* 2002, Ribaut *et al.* 2002). These studies evaluated the efficiency of MAS as well as the ability to transfer genes/QTLs from one genetic background to another (Frisch *et al.* 1999, Frisch and Melchinger 2001). These studies evaluated the population sizes required to carry out MAS successfully (Bonnett *et al.* 2002, Chao and Ukai 2000, Moreau *et al.* 1998).

This master's research was part of a larger collaborative, ACIAR funded project (Wheat Improvement in Sichuan Province: Application of Modern Breeding Technologies) in which I was employed as a research assistant. The ACIAR project combined the expertise of a number of scientists from the University of Southern Queensland, the Queensland Department of Primary Industries & Fisheries – Leslie Research Centre, the University of Adelaide, Waite Campus and the Crop Research Institute within the Sichuan Academy of Agricultural Sciences (SAAS) located in Chengdu, China. This project ran from July 1998 to April 2006 and was initiated to investigate a number of key areas in wheat breeding important to Australia and the Sichuan Province, China. There are a number of factors that influence the wheat production and quality in Sichuan and have been identified as necessary areas of research. The project concentrated on the following areas that were limiting yield and production of wheat in Sichuan: increasing genetic gains, disease resistance, intermittent reproductive sterility, pre-harvest sprouting and wheat quality.

Quality of wheat grains and its end products is important to both countries and this project was successful in utilising the technologies present in Australia to assess and improved the quality of Chinese fresh noodles in China. This research focussed on identifying effective methods to develop wheat varieties exhibiting high levels of noodle quality. To enhance this quality improvement, molecular markers were used to assess quality attributes in the germplasm grown in Sichuan. Information and germplasm gained through this ACIAR project enabled this current study to be carried out, also highlighting the potential to further improve Chinese varieties in the areas of colour and colour stability through the use of Marker-assisted selection.

This current research was carried out on populations that were generated through the original ACIAR project combining important germplasm from Australian and China. The initial crosses and first backcross was conducted by the Project Officer Dr. Eric Storlie in 2001. The varieties selected for these populations were the Australia varieties: Sunco and Tasman, and the Chinese varieties: Chuanmai 22 and Mianyang 11.

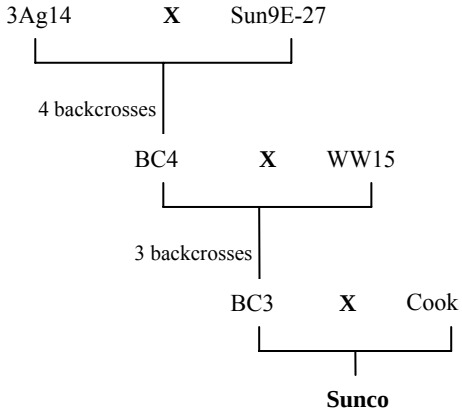
The Australian varieties were selected for a number of reasons, firstly because they are used often in wheat breeding and therefore are known to perform well. They also contribute to the traits being studied: Polyphenol oxidase (PPO) activity and Xanthophyll content. Pedigree's of the two Australian varieties; Sunco and Tasman (Figure 1-11), contain lines very important in Australia's wheat breeding history. Sunco includes the wheat variety Cook in its parentage, which was a wheat grown in Queensland in the late 1800's displaying a good milling quality and rust resistance (Spennemann 2001). Sunco has a quality classification of Australian Prime Hard, released in 1986 with resistance against stem rust, and performs with a moderate susceptibility to lead and stripe rust (NSW-DPI 2008). Sunco also has shown good flour and noodle colour quality (Mares and Campbell 2001) and therefore was an important inclusion to this study.

Tasman has a more complex lineage and includes lines from Mexico (CIMMYT – International Maize and Wheat Improvement Centre), Italy and South Africa. Tasman is classified as an Australian Hard wheat with a semi-dwarf stature, very strong straw and resistance against stem, leaf and stripe rust in Australia. Tasman contributes high levels of Xanthophyll content; however, it also contains an

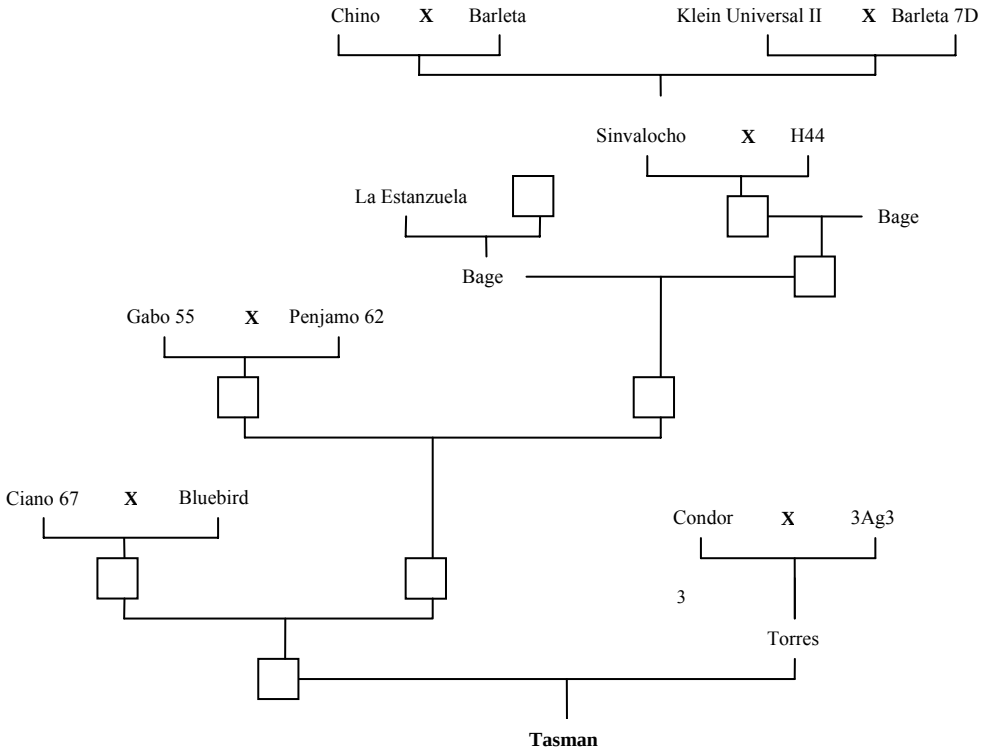
unfavourably high level of Polyphenol oxidase. Tasman was released as a variety in 1993 (Brennan *et al.* 1993).

Figure 1-11: Pedigree of parents; *Sunco* and *Tasman* selected for mapping population: Sunco x Tasman doubled haploid population *(Source: Kammholz et al. 2001)*

SUNCO



TASMAN



The Chinese varieties used in this study were developed in China's Zone V. Both varieties, Mianyang 11 and Chuanmai 22, have the variety Fan 6 as part of their pedigree. Fan 6 was the first landmark variety for Zone V in the 1970s and contributed greatly to wheat production and wheat breeding (He *et al.* 2001). A derivative of Fan 6, Mianyang 11 was developed by the Miangyang Prefectural Agricultural Research Institute (PARI) in 1979 and possesses the broad adaptation, high yielding potential, short stature and stripe rust resistance from Fan 6. In addition, Mianyang 11 has powdery mildew resistance, escape from head scab and early maturity. He *et al.* (2001) states "that it was the leading variety in the 1980s and contributed 1.5 million ha in 1984, out yielding Fan 6 by 11.3%."

Chuanmai 22 is also a derivative of Fan 6 and was developed from Mianyang 11/Chuanmai 20 in 1989 by the Sichuan Academy of Agricultural Sciences (SAAS). This improved variety maintains the resistance to stripe rust, powdery mildew and head scab, but has also showed tolerance to drought and poor soil fertility which are major concerns for the hilly cropping areas of the Sichuan basin of Zone V (He *et al.* 2001). The detailed pedigrees of Fan 6, Mianyang 11 and Chuanmai 22 have been displayed in Figure 1-12 and 1-13.

Figure 1-12: Pedigree information of Varieties Fan 6, *(Data source: He et al. 2001)*

i) Pedigree of Fan 6

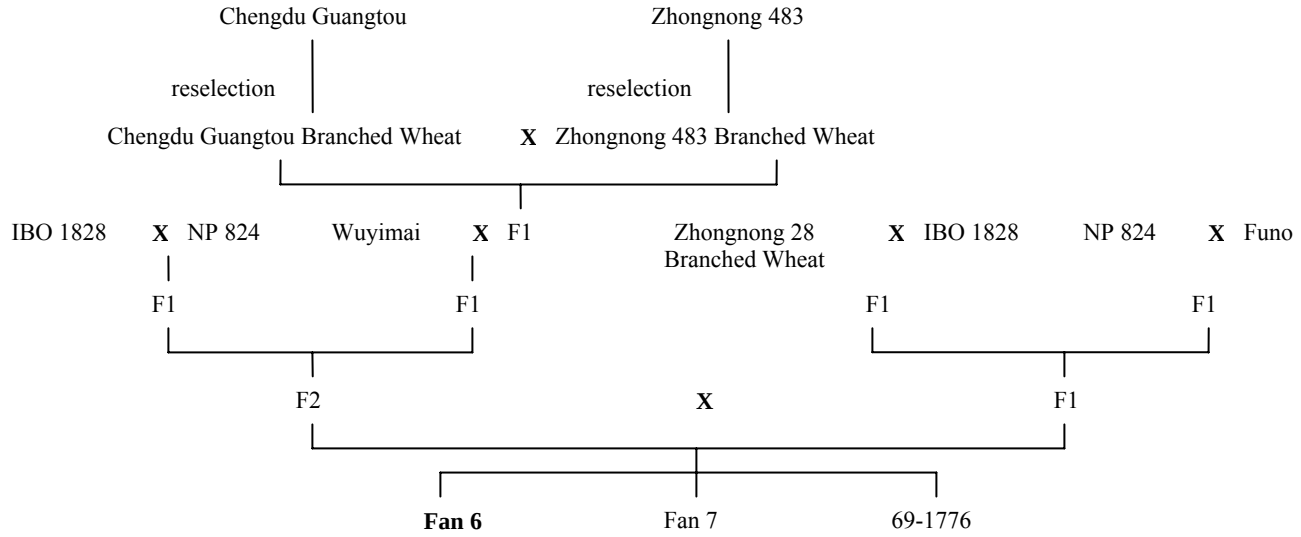
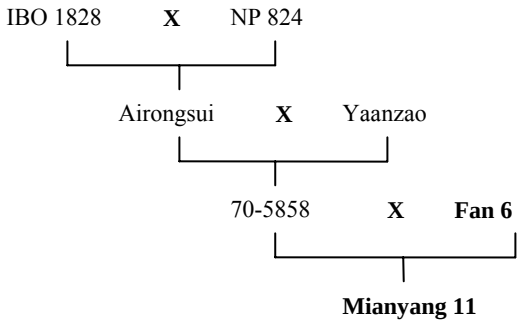
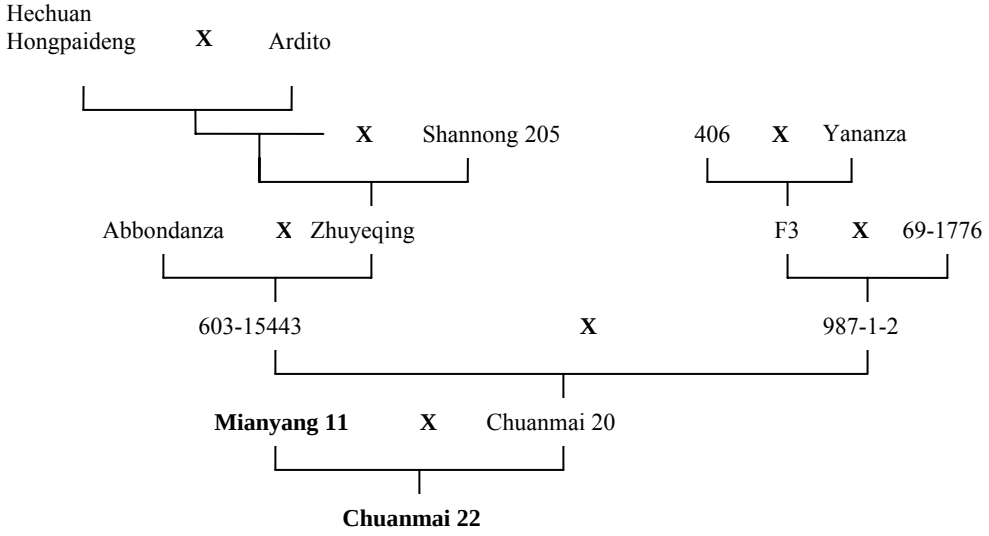


Figure 1-13: Pedigree information of Varieties Mianyang 11 and Chuanmai 22, *(Data source: He et al. 2001)*

ii) Pedigree of Mianyang 11



iii) Pedigree of Chuanmai 22



Specifically, the objectives of the study are:

1. To use the QTLs previously identified by Mares and Campbell (2001) to improve the flour colour characteristics in backcross populations generated between Australian varieties (Sunco and Tasman) and Chinese varieties (Chuanmai 22 and Mianyang 11).

2. To evaluate Marker-assisted selection as an efficient method of transferring QTLs located in Australian germplasm to a Chinese genetic background.

3. To test the usefulness of microsatellite markers as a tool to predict phenotype (Polyphenol oxidase activity and Xanthophyll content) in a novel genetic background.

4. To increase awareness and usefulness of molecular markers in wheat breeding in China.

Chapter 2: Establishment and Genotyping of Backcross Populations

2.1 Introduction

Backcrossing is a form of recurrent hybridisation, which integrates into an elite recurrent parent background specific alleles from donor lines while keeping the overall integrity of the recurrent parent intact (Poehlman and Sleper 1995). Using the Backcrossing method with in-breeding crops such as wheat, generates lines homozygous for particular traits in a relatively short period of time. If selection for the desired allele from the donor parent is conducted at each generation, a breeding program can introduce important genes from different sources and integrate them into highly adapted elite wheat varieties. Traditionally this selection of important alleles has been conducted phenotypically. In the past decade molecular markers have been successfully used to track the genetic sections from the donor parent, as well as select lines with a genome close to the recurrent parent (Langridge and Chalmers 2005). Combining traditional backcross methods with molecular markers has successfully introduced specific alleles for disease resistance, quality attributes

and many other traits (Frisch and Melchinger 2001, Frisch 2005, Fulton *et al.* 1997, Huang *et al.* 2004, Servin and Hospital 2002, Tanksley *et al.* 1996, Willcox *et al.* 2002, Yousef and Juvik 2002).

Including marker assisted selection into the breeding process will only quicken the process and decrease the number of lines requiring phenotyping (Bonnett *et al.* 2005). Marker Assisted Selection (MAS) can be particularly useful when the markers employed are co-dominant and reveal heterozygous individuals.

PCR and DNA sequencing have been integrated into traditional breeding programs to assist the identification of important traits in the wheat genome (Jones *et al.* 1997). A number of different molecular markers have been used in plant research, of which have been described in Chapter 1. A very important aspect of molecular marker use is their reproducibility in different genetic backgrounds and by different laboratories and researchers. The study by Jones *et al.* (1997) considered this aspect of the marker technology, comparing RAPD, AFLP and SSR markers. He concluded that RAPD markers were initially easy to perform, but the reproducibility of these markers was poor. Alternatively, the AFLP and SSR markers produced reliable and robust genotyping between different laboratories in Europe. Technical aspects of

markers also have to be considered. This includes the optimisation of chemicals, enzymes, DNA template and thermocycling machines used in the process.

In this current study SSR markers were employed due to their robust, co-dominant nature, wide availability and ease of use with backcrossing strategies (Korzun 2003).

This chapter will describe the process undertaken to generate the backcross populations used in this project. A genotypic evaluation of the four populations has been carried out to provide all the necessary information to complete the aims of this project.

2.2 Materials and Methods

Establishment of Backcross Populations

The parental material used in the production of the backcross populations were two Australian varieties: Sunco and Tasman, and two Chinese varieties:

Chuanmai 22 and Mianyang 11. The initial F_1 crosses and the first backcross were made by Dr. Eric Storlie in 2001, then as a postdoctoral fellow at the University of Southern Queensland, before the commencement of this project. These four populations were Sunco x Chuanmai 22, Sunco x Mianyang 11, Tasman x Chuanmai 22 and Tasman x Mianyang 11.

Both the first and second backcross, of the four populations, were carried out using the Chinese variety as the recurrent parent. After the backcrossing, individuals within each of the four populations went through three further generations of self-fertilisation (Single Seed Descent) before lines from each population were planted in a field trial. After each generation each line produced a different yield of grain, therefore the numbers of seed available for further plantings varied. The amount of seeds (of individual lines) planted in the three single seed descent generations, ranged from a minimum of 6 seeds to a maximum of 34 seeds. Between the first and second backcrosses, molecular markers were used to select the lines that would undergo the second backcross. During the second backcross the important lines, those carrying the donor marker alleles, were given priority. The backcrossing continued until there was no more viable pollen available. These lines were genotyped again at the

second and third generation of self-fertilisation. This process has been displayed in Figure 2-1.

All stages of population development took place in the Glasshouse facilities at the University of Southern Queensland (USQ).

QTL analysis and Molecular Marker Selection

The initial QTL analysis was carried out using the Sunco x Tasman linkage map (genotypic data) and the available phenotypic data (Mares and Campbell 2001). This linkage map was developed through GRDC funding and as an integral part of the Australian Winter Cereal Molecular Marker Program (AWCMMP). The phenotypic data used for this QTL analysis included Polyphenol oxidase (PPO) and Xanthophyll evaluation of Sunco x Tasman population material planted at Narrabri, NSW in 1998. This phenotypic data was collected and analysed by Daryl Mares (then at the University of Sydney).

A second round of QTL analysis was carried out after a refinement of the Sunco x Tasman linkage map. This was done through interval mapping analysis using the Q-gene software, version 3.04 (Nelson 1997). The revised

Sunco x Tasman linkage map can be viewed at the following website: http://cbbc.murdoch.edu.au/cmap.

The recent genotyping and map refinement on the Sunco x Tasman population was conducted by Dr. Anke Lehmensiek of USQ whereby a greater range of SSR markers were mapped to the areas of interest. These areas were located on chromosomes 2A, 2D, 3B and 7A. During this revision, all the markers were re-ordered and apparent double recombinants were removed. The refinement process is described fully in Lehmensiek *et al.* (2005).

Plant Material and DNA Extraction

DNA was extracted from plant material collected from four week old seedlings. For the first three screenings, the plant material was collected from seedlings in the USQ glasshouse.

The first genotyping of the four backcrossed populations was carried out in 2002 on 246 BC_1F_2 seedlings. At this stage the Phenol/Chloroform DNA extraction method was used. The genotyping carried out in 2003 was

conducted on BC_2F_3 seedlings using a kit supplied by Qiagen Pty Ltd. This method was used to extract genomic DNA from a total of 216 lines (over the four backcrossed populations). The decrease in total of lines (from 246 – 216) was due to the selection of lines (using molecular markers) between the first and second backcross. The Wizard Genomic DNA Extraction Kit was the method used in 2004 and 2005. This method allows tubes or microplates to be used. Tubes were used in 2004 on BC_2F_4 seedlings and the plate system was used in 2005 on BC_2F_5 seedlings. DNA was extracted from all four backcrossed populations, with 216 lines extracted in 2004 and 384 lines in 2005.

Full descriptions on all DNA extraction techniques are present in the Protocol Section in Appendix A.

Field trial of BC_2F_5 material

Lines selected after the 2004 genotyping were planted in a "Latinised row-column" designed field trial conducted at the Leslie Research Centre (QDPI & F). The field trial statistical design was developed by statistician Allison Kelly of QDPI & F. Each line was replicated three times, planted in a 0.5m row with

6 seeds per row. In the instances that there was not enough seed for a particular line, two replicates were planted and the third was substituted with the parental line, Sunco. All parental lines (Sunco, Tasman, Chuanmai 22 and Mianyang 11) and biochemical standard lines (Krichauff and Batavia) were planted within the trial site, replicated a total of nine times.

During the field trial, plant material was sourced from each genotype at various stages of development. Leaf material was taken from the first and the third (four week old) seedling of each row. Genotyping was carried out on the first seedling material only.

Once the plants had reached maturity the first plant from each row was harvested separately. The remaining five plants were bulked together. The grain was removed from the bulked samples at the Leslie Research Centre (LRC) using the large threshing machines. These machines remove and separate the grain from the wheat heads. The grain from these bulked samples was stored in humidity and temperature controlled units at the LRC. The single (first) plants were bagged and transported back to the USQ. The seed was then removed from the wheat heads by hand and stored for use in the biochemical tests (see Chapter 3).

Genotyping of lines produced in the four backcrossed populations

Genotyping was carried out at four different times throughout the project. It was very important to track the genotype at each selected marker locus from under the four different QTLs (PPO: 2A and 2D; Xanthophyll: 3B and 7A) highlighted in the rationale section of Chapter 1.

The SSR primer sequences were obtained from the Wheat Microsatellite Consortium (WMC), Agrogene (Röder *et al.* 1998), Pestova *et al.* (2000) and Graingenes (http://wheat.pw.usda.gov). The amplification of the SSRs used in this project was carried out in a PTC-100 thermocycler (Biometra). The samples for amplification contained approximately 20ng of wheat genomic DNA, 5µM each of the forward and reverse primer, 100µM of each dNTP, 1.5mM $MgCl_2$ and 0.5U Taq DNA polymerase in a total volume of 10µl. The following PCR profile was used on all samples: 1 cycle of 94^{o}C for 3 mins, followed by 40 cycles of 94 ^{o}C for 30secs, 50-60 ^{o}C (depending on the primer combination) for 30 secs, 72 ^{o}C for 1 min, and 1 cycle of 72 ^{o}C for 10mins. The amplified products were electrophoresed on 6% polyacrylamide gel using the Bio-rad Sequencing Gel Electrophoresis System, or on a 5.6% polyacrylamide

gel using a Corbett Robotics Gel Scan 2000. The single AFLP used in this project was carried out using the methods described in Chalmers *et al.* (2001).

The molecular markers (SSRs and AFLP) used throughout the genotyping process changed between the years 2002 and 2003. This occurred because more closely linked SSR markers were mapped to the revised Sunco x Tasman population (Lehmensiek *et al.* 2005). These differences are displayed in Table 2-1. The genotyping carried out in 2002 enabled lines containing the Sunco or Tasman allele to be selected for the second round of backcrossing. Likewise in 2003, all of the lines for each population were genotyped again to track the lines heterozygous or homozygous for the Australian parent alleles. The genotyping in 2004 was an important step in selecting lines for the field trial.

A number of lines were selected, containing different combinations of Australian and Chinese alleles. The complete list of these selected lines can be found in Table 2-3.

Table 2-1: Molecular Markers used in the genotyping of plant material in all four backcross populations – comparing years 2002 with 2003-2005.

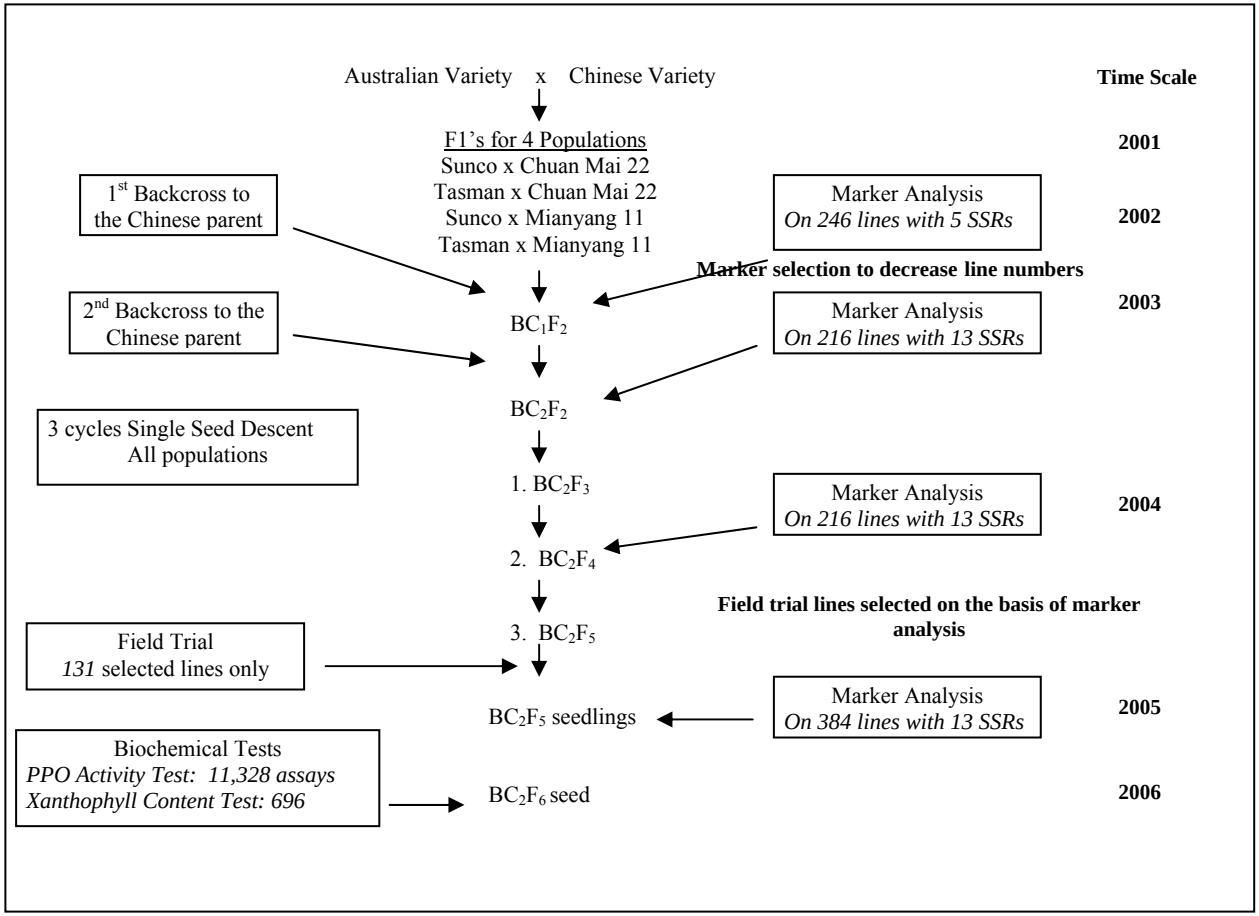
Population	**PPO - 2A**		**PPO – 2D**		**Xanthophyll – 3B**		**Xanthophyll – 7A**	
	2002	2003-2005	2002	2003-2005	2002	2003-2005	2002	2003-2005
Sunco x Chuanmai 22	-	*gwm372 gwm526*	*wmc18*	*wmc18 gwm157 cfd233 gwm301*	*gwm285 P36/M35*	*gwm285 gwm566*	*wmc17 wmc346*	*wmc17 wmc346*
Sunco x Mianyang 11	-	*gwm372 gwm526*	*wmc18*	*wmc18 gwm157 cfd233 gwm301*	*gwm285*	*gwm285 gwm566*	*wmc17 wmc346*	*wmc17 wmc346*
Tasman x Chuanmai 22	*wmc170*	*gwm372 wmc170 gwm526*	*wmc18*	*wmc18 gwm102 wmc181 gwm349 gwm301*	*gwm285*	*gwm285 gwm566 gwm108*	*wmc17 wmc346*	*wmc17 wmc346*
Tasman x Mianyang 11	*wmc170*	*gwm372 wmc170 gwm526*	*wmc18*	*wmc18 gwm102 wmc181 gwm349 gwm301*	*gwm285*	*gwm285 gwm566 gwm108*	*wmc17 wmc346*	*wmc17 wmc346*

2.3 Results

The process by which this study was carried has been depicted diagrammatically in Figure 2-1. It allows the process to be visualised as a whole, displaying the total volume of work conducted in the laboratory, glasshouse and the field.

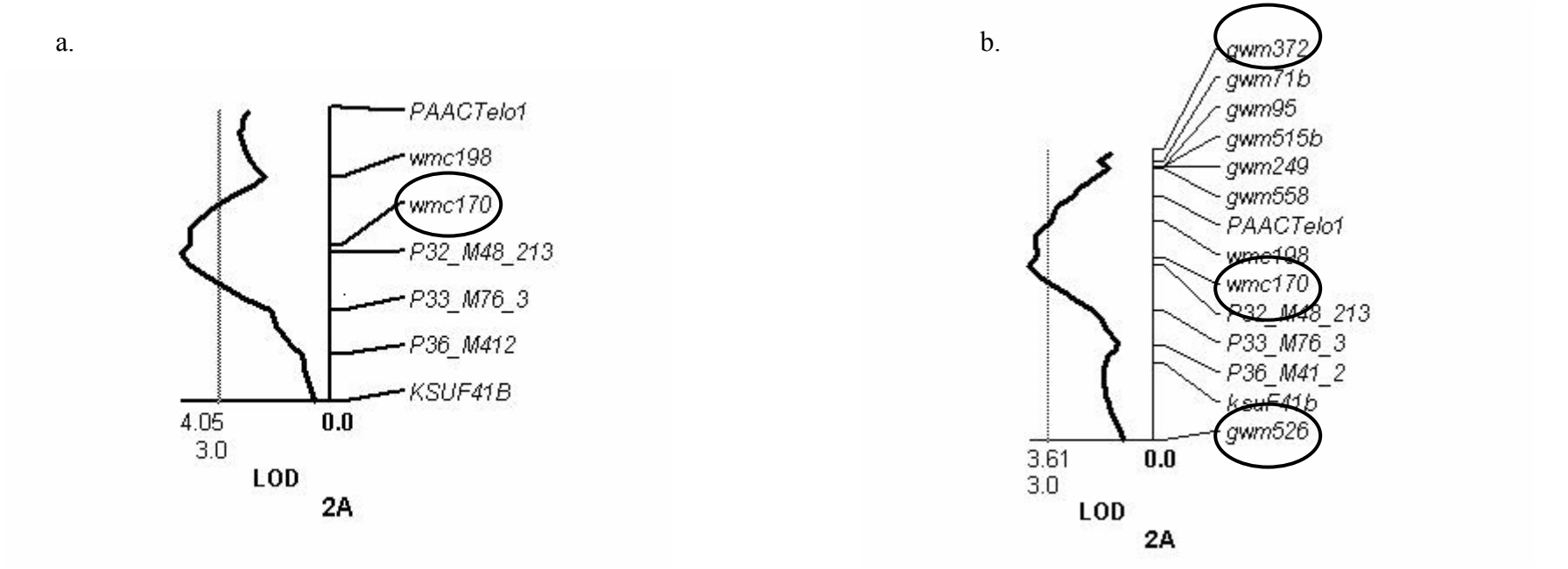
The lines carried through the backcrossing stage were selected by markers identified through QTL analysis. The genotypic and phenotypic data generated by previous research (Mares and Campbell 2001) allowed the re-identification of the QTLs for Polyphenol oxidase activity (PPO) and Xanthophyll Content on chromosomes 2A, 2D (PPO) and 3B and 7A (Xanthophyll).

Figure 2-1: Flow chart of laboratory and field work for the phenotyping and genotyping of the lines in the four backcrossed populations



The identification of these QTLs was performed twice, firstly with the original Sunco x Tasman linkage map and then again with the revised and curated version. The QTL traces using the original map have been displayed in section (a) of Figures 2-2, 2-3, 2-4 and 2-5. The section (b) of the Figures 2-2, 2-3, 2-4 and 2-5 contain the QTL traces generated with the revised Sunco x Tasman linkage map.

Figure 2-2: QTL analysis carried out on chromosome 2A of Sunco x Tasman DH population. All analysis used phenotypic data collected in the 1998 field trial; a. used the original genetic linkage map, b. used the revised linkage map.



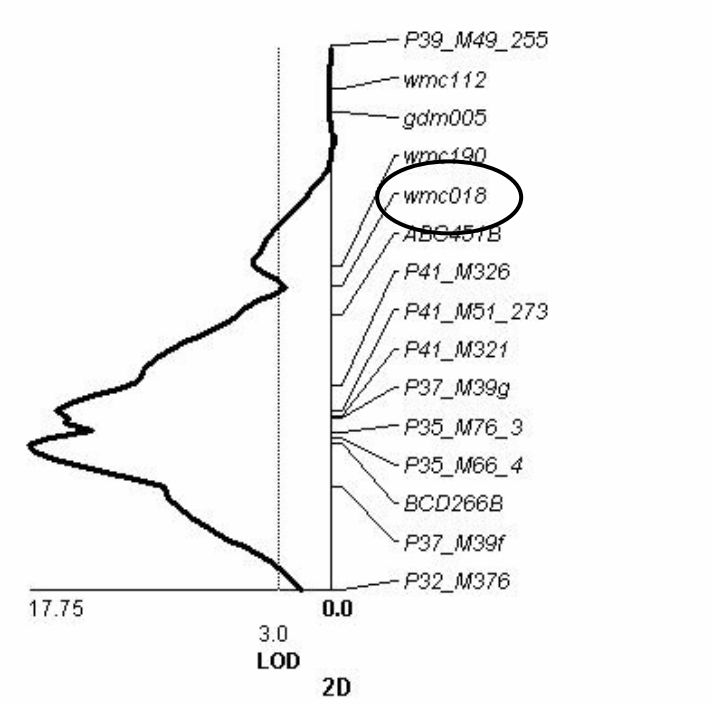
a. Analysis carried out in 2002 using the original linkage map

b. Analysis carried out in 2003 using the revised linkage map

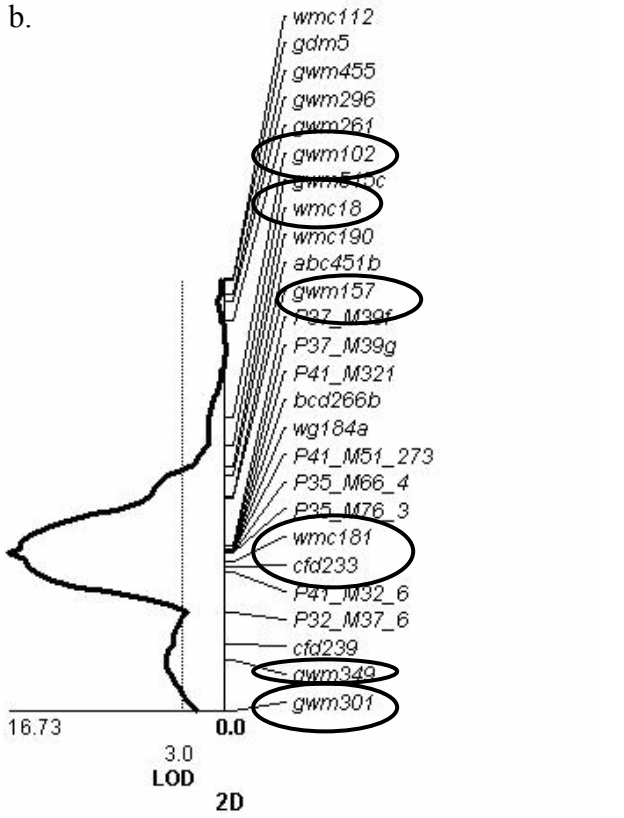
Legend: the circled markers on the QTL trace indicate the Microsatellite markers selected and used in the genotyping of the four backcross populations

Figure 2-3: QTL analysis carried out on chromosome 2D of Sunco x Tasman DH population. All analysis used phenotypic data collected in the 1998 field trial; a. used the original genetic linkage map, b. used the revised linkage map.

a.



b.

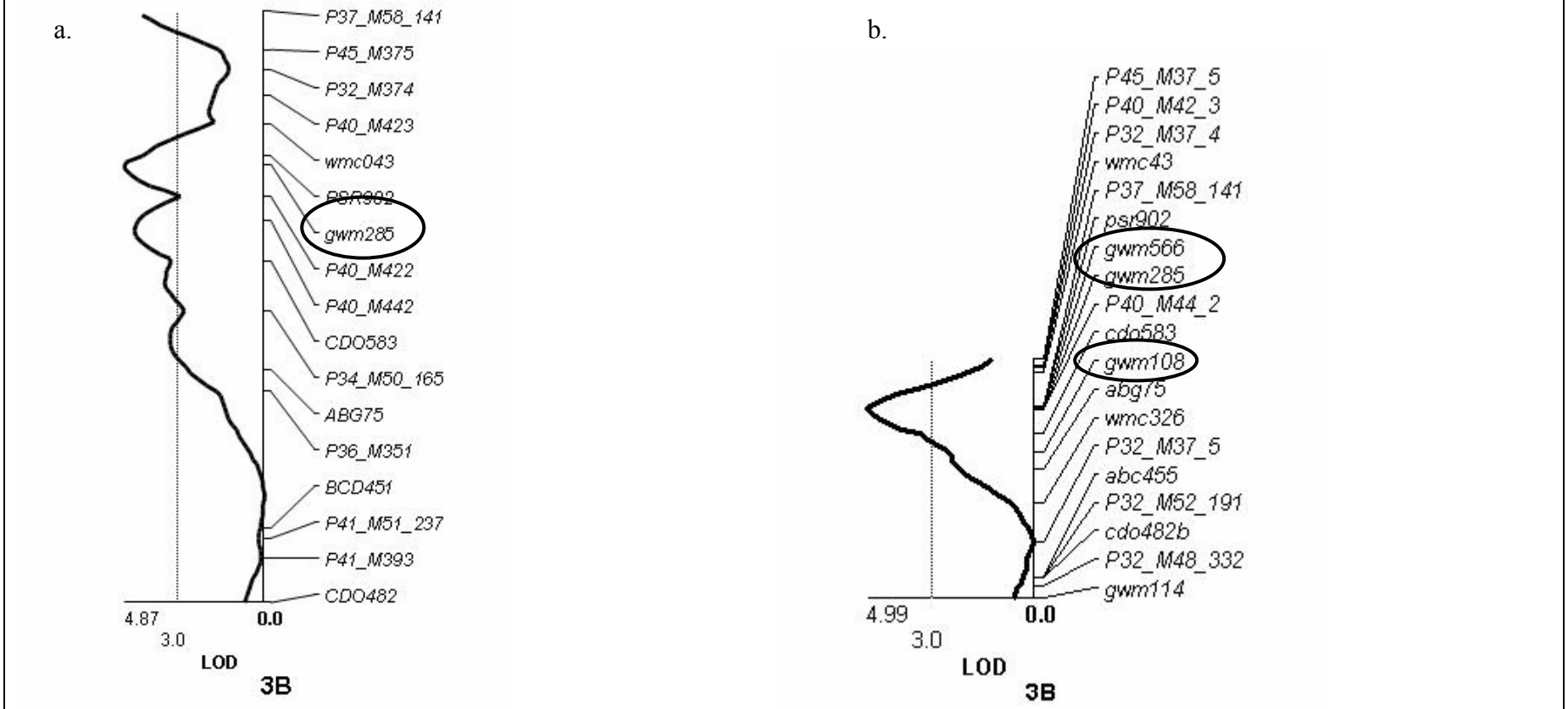


a. Analysis carried out in 2002 using the original linkage map

b. Analysis carried out in 2003 using the revised linkage map

Legend: the circled markers on the QTL trace indicate the Microsatellite markers selected and used in the genotyping of the four backcross populations

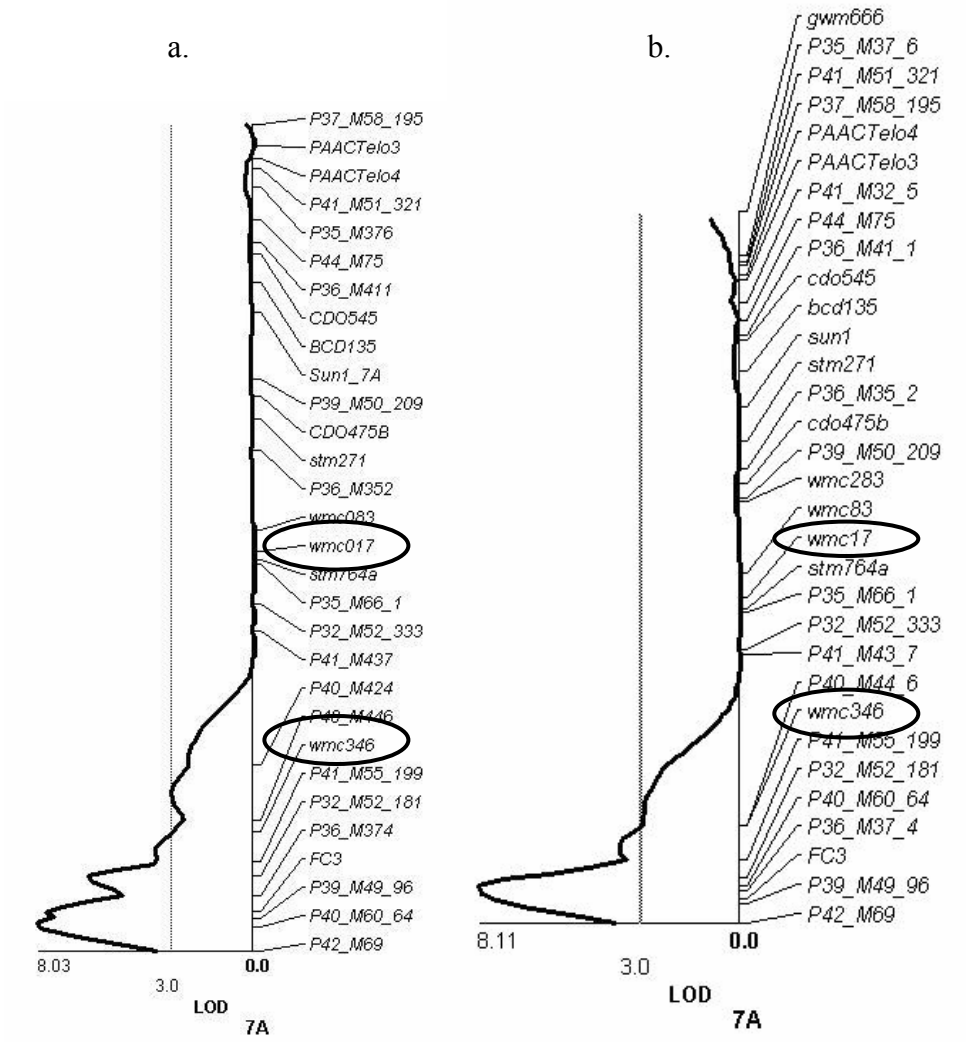
Figure 2-4: QTL analysis carried out on chromosome 3B of Sunco x Tasman DH population. All analysis used phenotypic data collected in the 1998 field trial; a. used the original genetic linkage map, b. used the revised linkage map.



a. Analysis carried out in 2002 using the original linkage map **b.** Analysis carried out in 2003 using the revised linkage map

Legend: the circled markers on the QTL trace indicate the Microsatellite markers selected and used in the genotyping of the four backcross populations

Figure 2-5: QTL analysis carried out on chromosome 7A of Sunco x Tasman DH population. All analysis used phenotypic data collected in the 1998 field trial; a. used the original genetic linkage map, b. used the revised linkage map.



a. Analysis carried out in 2002 using the original linkage map

b. Analysis carried out in 2003 using the revised linkage map

Legend: the circled markers on the QTL trace indicate the Microsatellite markers selected and used in the genotyping of the four backcross populations

The refinement of the Sunco x Tasman linkage map allowed a greater number of microsatellite markers to be used in the genotyping of the backcross populations. Table 2-2 displays work carried out on this linkage map and the benefit gained through the map curation.

Table 2-2: Summary of the genotyping information of the revised and original versions of the Sunco x Tasman linkage map.

	Revised Map		**Original Map**			
	Markers (no.)	Map Size (cM)	Markers (no.)	Map Size (cM)	Double Recominants Removed (no.)	(%)
Sunco x Tasman	345	2150	300	2920	635	1.03

(Source: Lehmensiek et al. 2005)

The volume of genotyping carried out changed substantially between the years 2002 and 2003. A total of five SSR markers were amplified on the 2002 lines. This was increased to ten SSR markers for the Sunco populations and thirteen SSR for the Tasman populations (2003 – 2005 lines). The number of SSRs used in the genotyping increased for all QTLs except the 7A Xanthophyll QTL. Extensive research was carried out to increase the number of SSR markers in this location, but the SSRs wmc17 and wmc346 were still the most closely linked microsatellites to this trait on 7A (Lehmensiek *et al.* 2005).

There were a wide range of lines included into this field trial: Ones that contained only the recurrent parent alleles; those that contained the donor alleles for one or more QTLs; those with alleles still remaining in the heterozygote state and those with a mixture of alleles. The complete range of lines, and total numbers, included into the field trial has been displayed in Table 2-3.

Table 2-3: Summary of the lines included in the 2005 field trial, A wide range of genotypes from each population were included to evaluate the use MAS.

	Chinese alleles only	Hetero-zygotes only	2A	2D	3B	7A	2A/ 2D	2A/ 3B	2A/ 7A	2D/ 3B	2D/ 7A	3B/ 7A	2D/3B /7A	2A/7A /3B	2A/2D /7A	2A/2D /3B	Mixture of the alleles
Sunco x Chuan-mai 22	0	6	2	5	6	1	1	5	0	2	1	6	1	2	1	2	0
Sunco x Mian-yang 11	9	5	3	2	4	6	0	2	1	1	0	4	1	1	0	0	2
Tasman x Chuan-mai 22	0	1	2	1	1	1	1	1	0	2	0	3	3	1	0	0	8
Tasman x Mian-yang 11	3	6	1	1	2	1	0	1	0	2	2	3	0	0	1	0	1
Total	**12**	**18**	**8**	**9**	**13**	**9**	**2**	**9**	**1**	**7**	**3**	**16**	**5**	**4**	**2**	**2**	**11**

Legend

*** 2A – PPO QTL; 2D – PPO QTL; 3B – Xanthophyll QTL; 7A – Xanthophyll QTL.**

Each column contains the number of lines with different genotypic combinations, focusing only on the markers used from each of the four QTLs for PPO activity and Xanthophyll content. The first column containing lines with only the Chinese parent alleles; second and last column with a combination of Chinese and Australian parental alleles; third to sixteenth column containing the Australian alleles for those particular QTLs.

2.4 Discussion

One of the aims of this project was to use previously identified QTLs from the Sunco x Tasman doubled haploid population and to subject them to Marker Assisted Selection. The field trial conducted was an important step in this research. It allowed a true indication of phenotype to be gained when the lines were subjected to environmental factors away from the semi-regulated environment of a glasshouse facility. The number and types of lines planted into this trial were selected purely on genotype. The purpose of leaving the phenotyping until the end of the process was to obtain some understanding on the use of molecular markers and their ability to predict a particular phenotype. A large range of genotypes were included into the field trial to generate an accurate view of the markers predictability and their use in wheat breeding situations. Some of these lines planted in the field trial may still be genetically segregating, as after two backcrosses and three generations of self-fertilisation with a degree of molecular marker selection, the lines will have 98.44% of alleles fixed. This will have to be taken into account when subjected to the biochemical testing.

The genotypic and phenotypic data collected for the Sunco x Tasman doubled haploid population was comprehensive enough to identify QTLs for both traits: PPO activity and Xanthophyll content. This information is the starting point for this project.

When attempting to use molecular markers in wheat breeding programs, they need to be user-friendly, economic and time efficient (Francia *et al.* 2005). AFLPs were used in the first phase of the AWCMMP as they produced a large number of polymorphic markers between the parents: Sunco and Tasman, allowing the linkage map to develop quickly. The markers produced through AFLPs are dominant markers and they do not have the ability to identify heterozygotes from homozygotes (Landjeva *et al.* 2007). Therefore the AFLPs in the original Sunco x Tasman linkage map were not useful to track the markers across the four backcross populations in this project and SSR markers were used instead.

It was very important to find reliable, polymorphic and co-dominant molecular markers on the areas of interest in the four chromosomes 2A, 2D, 3B and 7A of the Sunco x Tasman population. For Marker-assisted selection to be successful, the two flanking markers need to be tightly linked to the trait/gene of interest

(Babu *et al.* 2004, Francia *et al.* 2005). Therefore the successful refinement of the Sunco x Tasman linkage map allowed more tightly linked SSR markers to be used as flanking markers for the selection steps in this project. If the markers used in the 2002 genotyping session were used throughout the entire process, many positive lines would have been lost and negative lines could have been carried through without any information on possible recombination (Holland 2004).

Comparing the QTL traces generated for PPO activity and Xanthophyll content highlights the benefits of map curation. The QTL peaks from the revised linkage map are more sharply located on all four different chromosomes. This results was also found by Lehmensiek *et al.* (2005) on other linkage maps (CD87 x Katepwa; Cranbrook x Halberd).

There was a fall in LOD score for the PPO QTLs, whereby 2A went from LOD of 4.05 – 3.61 (Figure 2-2) and similarly for the 2D QTL, the LOD score went from 17.75 – 16.73 (Figure 2-3). Linkage map refinement is generally associated with an increased LOD score, as found in the 3B and 7A Xanthophyll QTL (Lehmensiek *et al*. 2005)

The slight drop in LOD score for the 2A and 2D PPO QTLs may indicate that the original QTL significance was over estimated. The addition of the reliable SSR markers underneath these QTL peaks has provided a more accurate measure of the QTL's significance. Therefore with these significant LOD scores and the re-identification of the QTL peaks, choosing markers from underneath these peaks would be the most suitable for MAS.

Chapter 3: Phenotyping through Biochemical Testing

3.1 Introduction

Breeding for quality attributes can be a difficult and time consuming task, but it is core to all wheat breeding programs worldwide. The measurement of most quality traits requires significant testing and therefore restricts the progress of new and improved genotypes for wheat breeders (Gale 2005).

The Asian markets are very important to wheat production in Australia, as they are large importer of Australian grown wheat and one of the largest consumers of wheat worldwide. A high proportion of Asian noodles are produced using bread-wheat flour, and therefore particular quality characteristics are required for high quality noodle production. These attributes include appropriate flavour and texture (Fu 2008-in press), protein content, dough strength and starch pasting properties (Crosbie and Lambe 1993) and flour colour (Asenstorfer *et al.* 2006, Fu 2008-in press, Mares and Campbell 2001, Zhang *et al.* 2008).

The colour of the flour can be affected by a number of components; these being, the colour of the starchy endosperm, the presence of bran flakes from

milling and differing genetic backgrounds. Xanthophyll content is a large contributor to the yellowness of wheat flour, which in turn determines the colour of the noodles. This level of xanthophyll is variety dependent in wheat, and is present in Australian hard wheat varieties (Asenstorfer *et al.* 2006, Fu 2008-in press). A bright yellow colour is required of Yellow alkaline noodles, and if this can be delivered to the noodle through the flour colour, then only minimal amounts of alkaline salts are required for the production of these noodles (Zhang *et al.* 2008). In contrast, He *et al.* (2004) states a low yellow pigment level is required for Chinese white noodles, where a bright white noodle colour is required for Chinese noodles.

Colour stability is also a very important part of noodle production, and this colour is required to remain stable for 24-48 hours after preparation. A high level of Polyphenol oxidase (PPO) activity in wheat flour has been linked to darkening of fresh noodles (Asenstorfer *et al.* 2006, Mares *et al.* 1997). Levels of PPO activity present in the wheat grain can be calculated through a whole seed assay (Bernier and Howes 1994). This allows breeders to phenotype important breeding lines and to assist in identification of the genomic areas responsible for this trait. These characteristics of noodle quality were discussed fully in Chapter 1.

An aim of this project is to generate wheat lines with enhanced quality attributes. As a general rule the Chinese varieties contribute a low level of PPO activity and Xanthophyll content, therefore including the Sunco alleles for these traits should only benefit the quality of these wheat lines. QTLs for high PPO activity (contributed by Tasman) and high levels of Xanthophyll content contributed by Tasman on 7A and to a lesser degree by Sunco on 3B, have been located in the Sunco x Tasman population (Mares and Campbell 2001); therefore transferring the Sunco alleles within these genomic areas into the Chinese background may enhance the whiteness of flour colour and the colour stability of the noodles produced. To research this theory high quality phenotypic data needs to be generated using the methods used in current breeding programs. Testing for PPO activity and Xanthophyll content are time consuming assays; therefore identifying molecular markers associated with these traits would benefit further molecular research and breeding endeavours.

3.2 Material and Methods

The grain produced from the field trial (Chapter 2) was retained for biochemical testing. Both tests were carried out in the laboratory of the University of

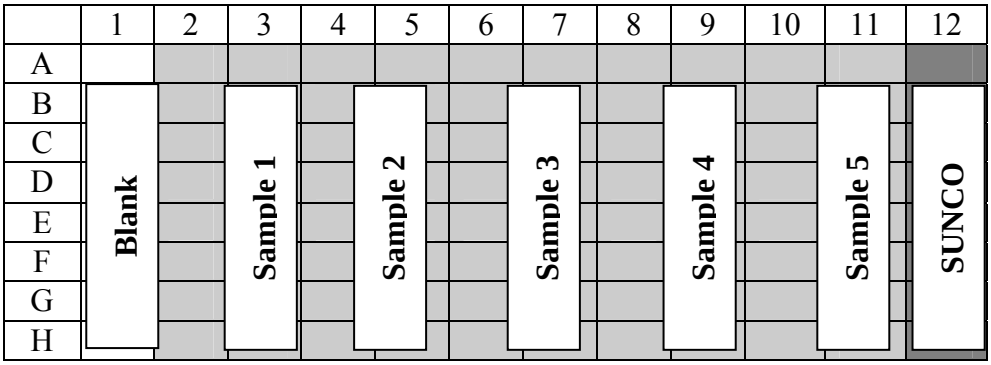
Southern Queensland using laboratory statistical designs developed in consultation with statistician Allison Kelly. The design included all test samples from the field trial, parental material (Sunco, Tasman, Chuanmai 22 and Mianyang 11) and the biochemical standards (Batavia and Krichauff). The laboratory designs were generated specifically for each biochemical assay, taking into account the number of samples tested at the one time, this being five samples for the PPO assay and eleven samples for the Xanthophyll assay. The randomized design also incorporated duplication of test samples to remove any laboratory or field effects from the analysis.

Polyphenol Oxidase Activity of Single Seeds

The method used to complete this testing was a modified version of the original single seed assay by Bernier (1994), which is based on the reaction of PPO with the substrate tyrosine. The modifications made to the original method were carried out by Dr Daryl Mares (Mares and Panozzo 1999) to optimize the assay for Australian germplasm and conditions. This modified method is currently used widely throughout Australian wheat breeding programs.

This assay is carried out in a microplate separating the samples into three distinct areas. The first row always served as the reagent blank. Rows 2 – 11 contained the field trial genotypes, and the last row always included Sunco material, the internal standard. Single seeds were placed into each well of a microplate except for the first row. Five field trial genotypes were tests on each plate, and seeds from each of these genotypes were placed in two rows each. This assay was conducted over a number of days. The first plate analysed each day consisted of all parental and biochemical standard varieties. These varieties included Tasman, Chuanmai 22, Mianyang 11, Batavia, Krichauff and Sunco. The microplate design for the PPO assay is displayed in Figure 3-1.

Figure 3-1: Design of the microplate used for each PPO assay carried out on the field trial material



Tyrosine solution (200µl) was added to each well of the plate using a multi-channel pipette without the addition of bubbles to the well. The tyrosine stock solution recipe and reagents necessary for this assay are displayed fully in Appendix A.

Each plate was covered and incubated for 3 hours at 37^{o}C. After incubation, 100µl of the substrate was removed from each well with a multichannel pipette and placed into a clean microplate to be read in a microplate reader at the optical density of 415nm. Each plate was assessed for outliers once all the optical densities for each well were calculated.

For each genotype analysed per microplate, a mean value for each of the 16 replicate results was calculated. The mean of the blank row was calculated and subtracted from each of the genotype means to produce a 'corrected' mean value for each genotype. For each sample, Standard Deviations and Standard Errors were calculated and outliers were removed if it produced a standard deviation of >0.05.

Frequency distributions of each individual population were carried out using the populations PPO activity results. The degree of skewness was calculated

for each of the four backcross populations through the computer program SPSS, version 15. This computer program calculates skewness using the below equation:

$$\left[\frac{\sqrt{n(n-}}{n-2} \right] \times \frac{m_3}{m_2^{3/2}}$$

Where

$$m_r = \sum \frac{(X^i - \bar{X}}{n}$$

Because of the lack of normality and the degree of skewness present in the frequency distributions, a general t-test could not be used; therefore the conservative Welch's t-test was used in further analysis. This test enabled comparisons to be made between the four parental varieties and to test for significant differences between transgressive segregants present in each population.

Xanthophyll Content from Flour Samples

Samples of grain from each genotype and parental variety grown in the field trial (Chapter 2) were used in this assay. Approximately 20 grams of field trial grain was milled to produce the flour samples required for the Xanthophyll content biochemical test. All milling was carried out by the staff of the Quality Laboratory at the Department of Primary Industries and Fisheries – Leslie Research Centre in a Quadrumat Junior Mill.

1 gram of flour was weighed into 50ml conical ended test tubes and then 5mls of methanol was added to each tube. All tubes were placed on their sides attached to the orbital shaker and gently mixed for 15 minutes. After being thoroughly mixed the tubes were centrifuged for 15 minutes at 4000rpm. The supernatant was then filtered through a 0.45µ syringe filter to ensure all flour particles were removed. The supernatant was then transferred into clean 15ml tubes and placed into a warm water bath to inhibit the samples becoming turbid before measuring the optical density of the extract at 436nm compared to a methanol blank.

Results from samples that were duplicated within the randomized statistical design were averaged to produce a single optical density result. Standard Deviations and Standard Errors were calculated for each sample or group of samples. If a particular sample mean possess a standard deviation of above 0.05 it was removed from the analysis.

Frequency distributions were carried out for Xanthophyll contents of each backcross population. The degree of skewness was calculated for each of the distributions using the SPSS program (v15) and the equation displayed above. Again the lack of normality in these distributions indicated the conservative Welch's t-test was required to perform the comparisons between parental Xanthophyll content data.

3.3 Results

Polyphenol Oxidase Activity

The PPO assays of lines from the four Australian x Chinese crosses resulted in the measurement of a total of 109 96-well microplates, together with nine

microplates of standards and parental varieties. This equates to 118 plates and a total of 11,328 assays analysed over nine days. An example of the PPO assay after the incubation period is displayed in Figure 3-2.

The number of tests carried out per day varied; however the consistency of the results between days was assessed by the number of outliers observed. These ranged from 5.63% to 8.48%. A number of outliers were removed from the raw data based on their standard deviations of the mean. 6.48% of the total assays conducted (734 assays of 11,328) were removed. The number of outliers observed and removed on the separate days of testing is listed in Table 3-1.

Figure 3-2: An example of a microplate of a PPO assay after the three hour incubation period. Refer to Figure 3.1 for sample placement pattern

Table 3-1: A summary of the outliers evaluated from each PPO assay conducted on the 2005 field trial material; genotypes of all four backcrossed populations, parental varieties and standards.

Day of PPO testing	Number of Outliers	Total Number of Assays	Percentage of Outliers / Total Assays
1	57	672	8.48%
2	92	1344	6.85%
3	81	1440	5.63%
4	93	1536	6.05%
5	83	1440	5.76%
6	94	1440	6.53%
7	85	1440	5.90%
8	33	576	5.73%
9	116	1440	8.06%
TOTALS	*734*	*11,328*	*6.48%*

Genotypes that produced results with a high standard deviation (>0.05) were looked at further. If the standard deviation wasn't lowered by the removal of the outliers these genotypes were repeated. Table 3-2 highlights the genotypes that produced initial high standard deviations, and compares their repeat results. These genotypes were found in all four backcross populations, with the most genotypes being from the Sunco x Chuanmai 22 and Tasman x Chuanmai 22 populations.

The frequency distribution of the final results for PPO activity is shown in the graphs of Figure 3-3 and Figure 3-4. These results display skewness to a higher

PPO activity, with the exception of the Sunco x Mianyang 11 population. The distributions also highlight a number of lines with lower PPO activity results than either parent. When the PPO activity data from all four populations were investigated further, a number of significant transgressive segregants were identified. Both of the Sunco populations contained 17 lines each producing significantly lower PPO activity levels than either parent (14.66% of the Sunco x Mianyang 11 population and 14.05% Sunco x Chuanmai 22 population). Of those lines, 5 lines from the Sunco x Mianyang 11 population and 6 lines from the Sunco x Chuanmai 22 population, produced PPO activity levels lower than Sunco. The level of PPO activity whereby the transgressive segregants become significantly different to either parent varies for each population. These levels were compared to the PPO activity levels produced by each parent, and are shown in Table 3-3. A PPO activity level (OD415nm) of 0.111 (Sunco x Mianyang 11 population) and 0.109 (Sunco x Chuanmai 22 population) is required to be significantly lower than the PPO level of Sunco.

The PPO activity data was analysed in a similar way for the Tasman populations, resulting in 3 lines from the Tasman x Mianyang 11 population and 10 lines from the Tasman x Chuanmai 22 population producing significantly lower PPO activity levels than either Chinese parent. Again,

different levels of PPO activity were found to be significantly lower than the Chinese parent in the Tasman populations. This being a PPO activity level (OD415nm) of 0.122 (Tasman x Mianyang 11 population) and 0.130 (Tasman x Chuanmai 22 population). The Tasman genome present in these populations inherently increases the PPO activity levels with; 26.47% (18 lines) of the Tasman x Mianyang 11 population, and 33.78% (25 lines) of the Tasman x Chuanmai 22 population producing PPO activity levels significantly higher than Tasman.

The descriptive information (means, standard deviations, sample size, standard error and margin of error) produced by the parental varieties, parental pairs and the entire populations can be found displayed in Table 3-3. Comparisons were made between each of the parental varieties using the conservative Welch's test, and it was found that there was a significant difference between the two Australian varieties, Sunco and Tasman. There was also a significant difference seen between Sunco and Tasman and both Chinese varieties, but there were no differences between the two Chinese varieties themselves.

Table 3-2: Summary of the genotypes reanalysed because of a high initial Standard Deviation

Genotype	Standard Deviation 1st assay	Standard Deviation Repeat assays
SC28-6	0.053	0.047; 0.044
SC33-4	0.062	0.039; 0.036
SC27-10	0.113	0.046
SC28-4	0.056	0.039; 0.042
SC32-2	0.057	0.036
SC10-9	0.059	0.049
SC32-1	0.061	0.048
SC27-3	0.053	0.042
SC10-11	0.117	0.033; 0.045 ; 0.038
SC28-8	0.158	0.030; 0.035
SC28-4	0.056	0.039; 0.042
SC33-4	0.062	0.039
SM57-14	0.056	0.029; 0.034
SM27-9	0.075	0.033
SM15-10	0.058	0.031; 0.031
SM15-10	0.058	0.031
TC28-8	**0.082**	**0.066**
TC28-11	0.056	0.034
TC35-7	0.058	0.049
TC31-6	**0.095**	**0.052**
TC28-8	0.075	0.049
TC31-11	0.056	0.038
TC31-6	0.064	0.038; 0.048
TC28-4	0.052	0.024; 0.035
TC28-4	0.052	0.024; 0.035
TC35-7	**0.094**	**0.063**
TC31-11	0.088	0.045
TM4-6	0.053	0.040; 0.039
TM28-1	**0.053**	**0.060**
TM31-11	**0.055**	**0.074**
TM29-5	**0.083**	**0.052**
TM29-6	0.099	0.027
TM20-2	0.076	0.037
TM29-15	0.054	0.039
TM29-5	0.055	0.031; 0.042
TM29-5	0.055	0.042

- bold text indicates lines with a standard deviation >0.050

Figure 3-3: Frequency distribution graphs of PPO activity (OD 415nm) results for each of the Sunco backcross populations

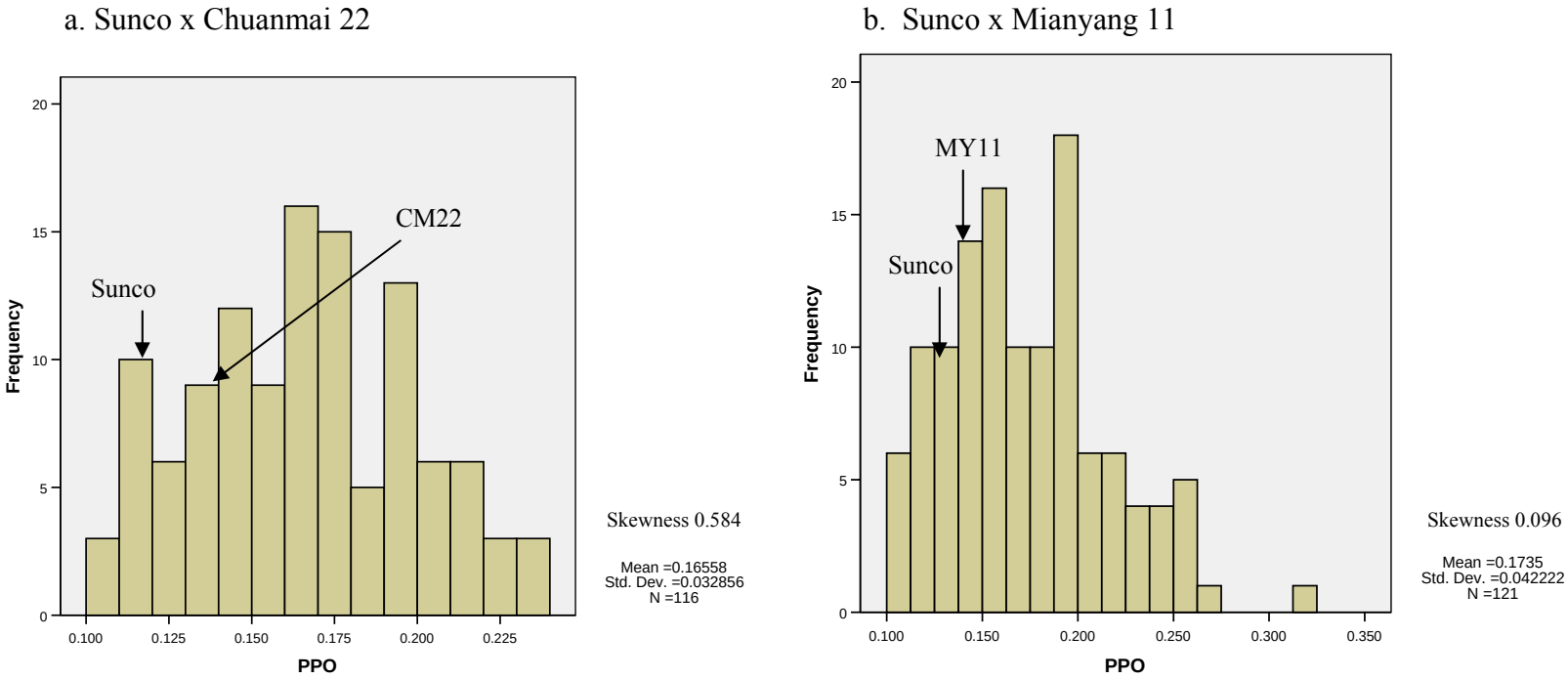
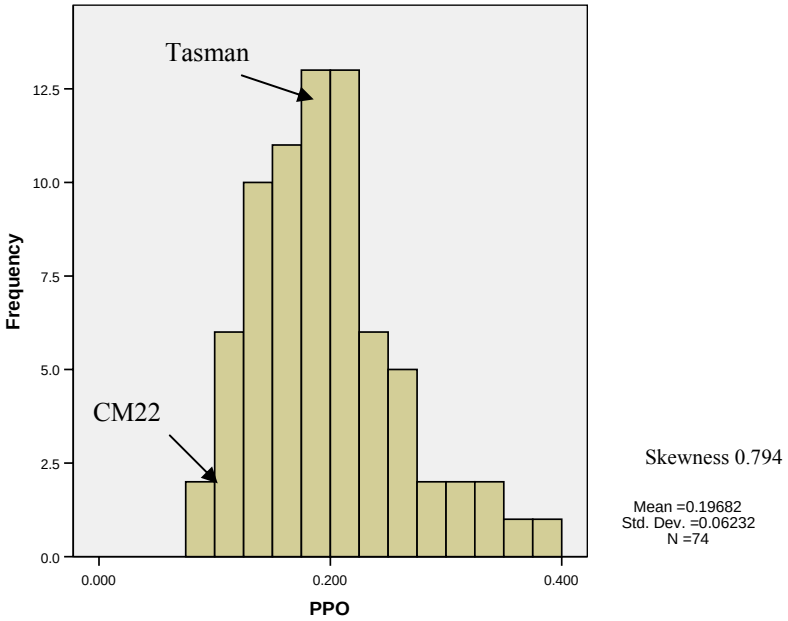


Figure 3-4: Frequency distribution graphs of PPO activity (OD 415nm) results for each of the Tasman backcross populations

a. Tasman x Chuanmai 22



b. Tasman x Mianyang 11

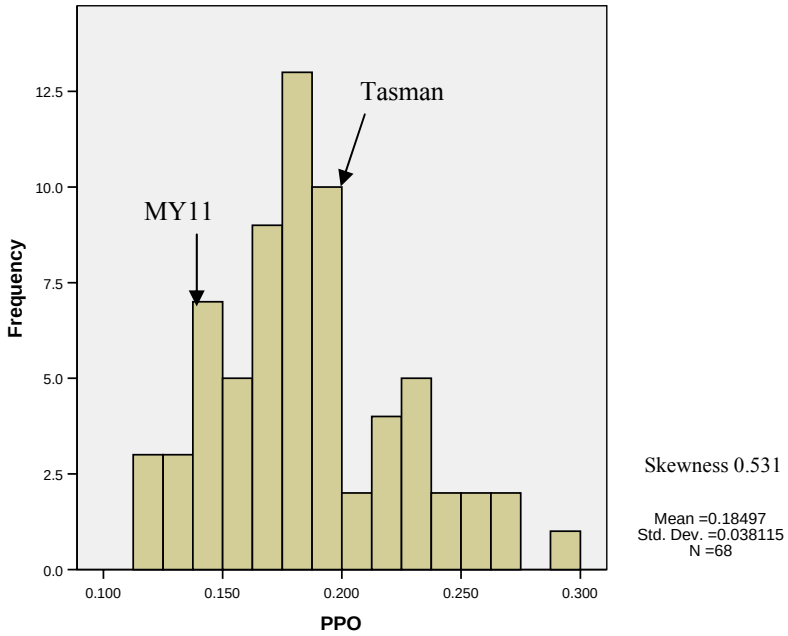


Table 3-3: Descriptive information for each backcross population for PPO activity

Individual Parents	Mean PPO activity OD415nm	Standard Deviation	Sample size (n)	Standard Error	Margin of Error (at 95%)
Sunco	0.121[a]	0.0196	118	0.0018	±0.0035
Chuanmai 22	0.143[c]	0.0151	13	0.0042	±0.0082
Mianyang 11	0.137[c]	0.0114	13	0.0032	±0.0062
Tasman	0.192[b]	0.0435	14	0.0116	±0.0228
Parental Pairs					
Sunco & Chuanmai 22	0.132	0.0160	2	0.0109	±0.0214
Sunco & Mianyang 11	0.128	0.0089	2	0.0063	±0.0124
Tasman & Chuanmai 22	0.167	0.0336	2	0.0237	±0.0465
Tasman & Mianyang 11	0.162	0.0401	2	0.0284	±0.0556
Population Data					
Sunco x Chuanmai 22 Pop[n]	0.172	0.0401	121	0.0037	±0.0072
Sunco & Mianyang 11 Pop[n]	0.166	0.0033	116	0.0031	±0.0060
Tasman & Chuanmai 22 Pop[n]	0.195	0.05919	74	0.0068	±0.01339
Tasman & Mianyang 11 Pop[n]	0.188	0.0392	68	0.0047	±0.0092

Table 3-3

Legend: *Individual Parents*: statistical data obtained for each parent separately.
Parental Pairs: statistical data obtained for each pair.
Population Data: statistical data obtained for each Backcross population generated in this project.

[a]: statistical difference between the mean of Sunco and all other parents
[b]: statistical difference between the mean of Tasman and all other parents
[c]: statistical difference between the mean of Chuanmai 22 and Mianyang 11 and Sunco and Tasman

Xanthophyll Content

The Xanthophyll testing of the field material consisted of 696 individual tests over seventeen different days. The raw data was assessed, and if particular tests for genotypes had a high standard deviation (<0.005), then those genotypes were repeated. The repeats were included in the complete data set if they lowered the standard deviation between the individual field replication, and genotype. For the data analysis, each genotype was assessed as a group of the three field replications and any duplication/repeats carried out in the testing process, this being termed 'genotype group' in the discussion. The numbers of individual line tests that were repeated have been summarized in Table 3-4. For some of the individual lines and genotype groups, the standard deviations could not be lowered with a repeat test.

Table 3-4: Summary of the results from the repeat xanthophyll content tests

Population	No. of Repeats (of total 100)	Result Class	Number of Repeats	Percentage of total repeats
Sunco x Chuanmai 22	**25**	**a.** Repeats corrected Std Dev in both individual line and genotype **b.** Repeats corrected Std-Dev in replicates of a individual line **c.** Repeats didn't correct Std Dev for individual line or genotype	**14** **12** **5**	**45.2%** **38.7%** **16.1%**
Sunco x Mianyang 11	**31**	**a.** **b.** **c.**	**17** **7** **1**	**68%** **28%** **4%**
Tasman x Chuanmai 22	**21**	**a.** **b.** **c.**	**16** **4** **1**	**76.2%** **19.0%** **4.8%**
Tasman x Mianyang 11	**23**	**a.** **b.** **c.**	**7** **8** **8**	**30.4%** **34.8%** **34.8%**

Frequency distribution curves were produced from the Xanthophyll content data for each backcross population (Figure 3-5 and Figure 3-6). The Sunco

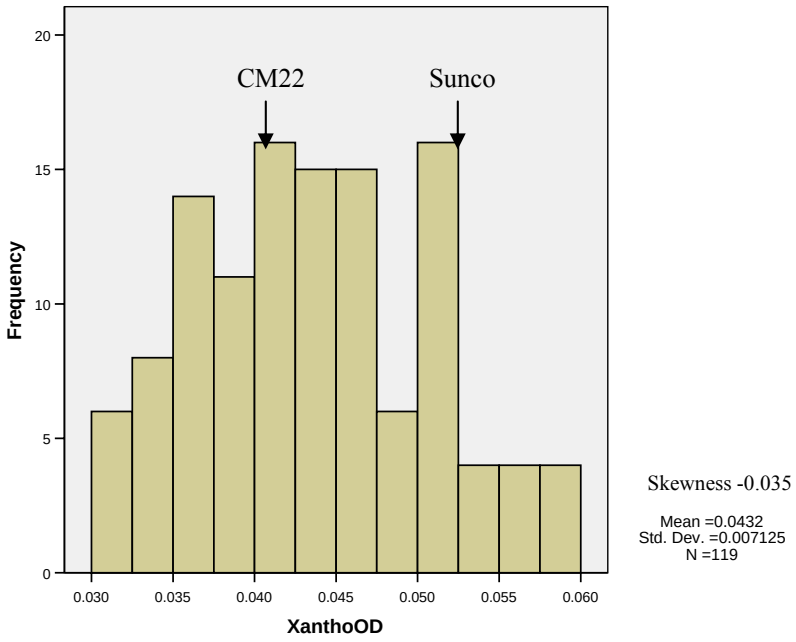
populations show low levels of skewness, while the Tasman populations show skewness towards high xanthophyll content values.

When the lines of the Sunco populations are considered individually, a very large proportion of lines performing better than Sunco and the Chinese parent are evident, therefore conferring a trend towards low levels of Xanthophyll content. 95.65% (Sunco x Mianyang 11) and 90% (Sunco x Chuanmai 22) of the lines had a lower Xanthophyll content level than Sunco. Similarly, 76.52% (Sunco x Mianyang 11) and 41.67% (Sunco x Chuanmai 22) had a lower Xanthophyll content level than the Chinese parent. The degree of significance for these trends has not been assessed due to the lack of multiple line testing carried out during this assay. To test Xanthophyll content single flour samples are tested, therefore not producing the multiple data points required of further data analysis. Also due to the nature of the statistical laboratory design for this assay not all lines were duplicated during the assessment of Xanthophyll content on each of the four backcross populations.

The descriptive information generated through the Xanthophyll content assay is displayed in Table 3-5.

Figure 3-5: Frequency distribution graphs of Xanthophyll content (OD 436nm) results for each of the Sunco backcross populations

a. Sunco x Chuanmai 22



b. Sunco x Mianyang 11

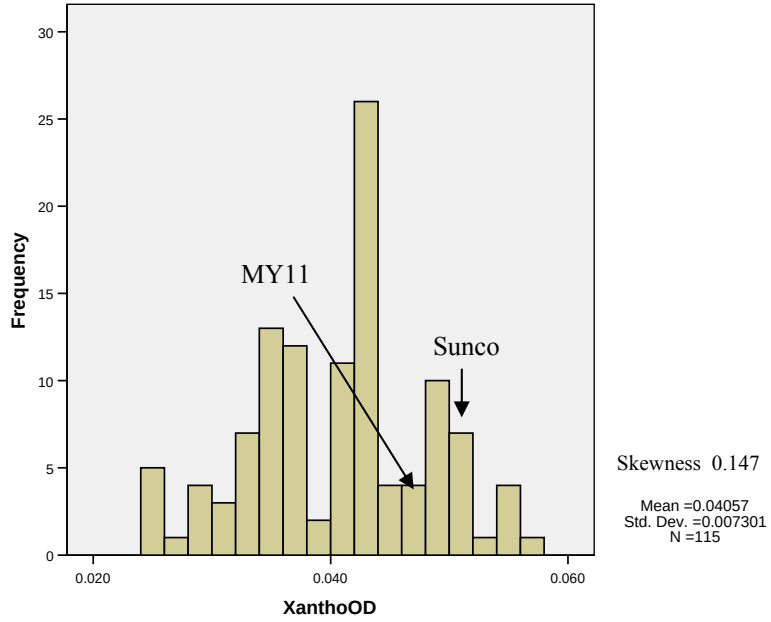
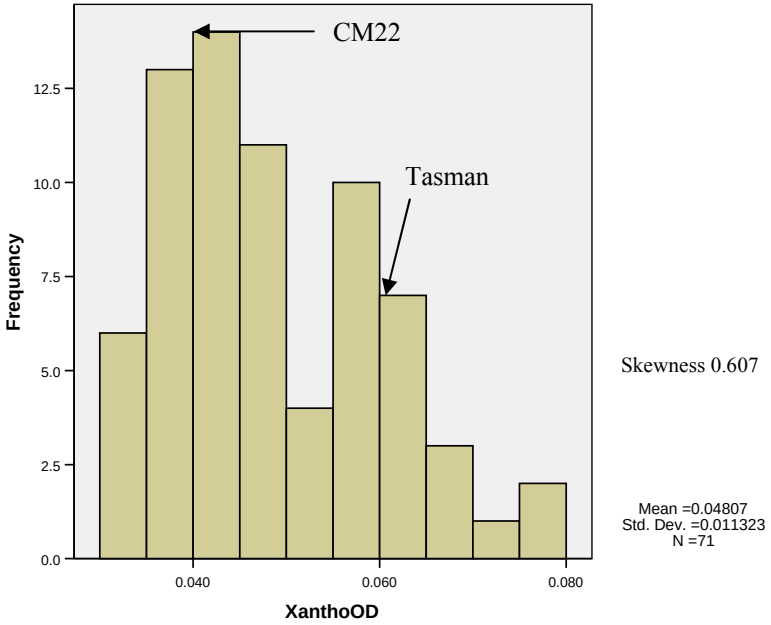
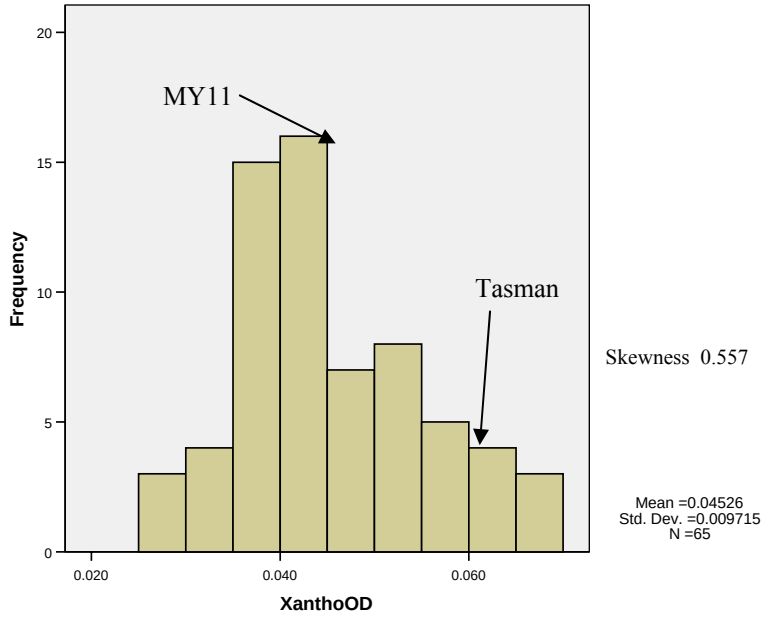


Figure 3-6: Frequency distribution graphs of Xanthophyll content (OD 436nm) results for each of the Tasman backcross populations

a. Tasman x Chuanmai 22



b. Tasman x Mianyang 11



Comparisons were made between the parental varieties with respect to their Xanthophyll contents, and the only significant difference seen was between the two Chinese varieties compared to the Australian varieties. There were no significant difference produced between Sunco and Tasman, or between Chuanmai 22 and Mianyang 11.

Table 3-5: Descriptive information for each backcross population for Xanthophyll content

Individual Parents	Mean Xantho-phyll Content OD436nm	Standard Deviation	Sample size (n)	Standard Error	Margin of Error (at 95%)
Sunco	0.052[a]	0.0053	56	0.0007	±0.0014
Chuanmai 22	0.041[c]	0.0066	3	0.0037	±0.0074
Mianyang 11	0.046[c]	0.0051	4	0.0026	±0.0050
Tasman	0.064[b]	0.0031	3	0.0006	±0.0035
Parental Pairs					
Sunco & Chuanmai 22	0.047	0.0080	2	0.0057	±0.0112
Sunco & Mianyang 11	0.049	0.0047	2	0.0033	±0.0065
Tasman & Chuanmai 22	0.052	0.0160	2	0.1133	±0.0222
Tasman & Mianyang 11	0.055	0.0126	2	0.0089	±0.0176
Population Data					
Sunco x Chuanmai 22 Pop[n]	0.043	0.0072	119	0.0007	±0.0013
Sunco & Mianyang 11 Pop[n]	0.041	0.0072	115	0.0007	±0.0013
Tasman & Chuanmai 22 Pop[n]	0.048	0.0113	71	0.0013	±0.0026
Tasman & Mianyang 11 Pop[n]	0.045	0.0102	65	0.0013	±0.0025

Table 3-5

Legend: *Individual Parents*: statistical data obtained for each parent separately.
Parental Pairs: statistical data obtained for each pair.
Population Data: statistical data obtained for each backcross population generated in this project.
[a]: statistical difference between the mean of Sunco and all other parents
[b]: statistical difference between the mean of Tasman and all other parents
[c]: statistical difference between the mean of Chuanmai 22 and Mianyang 11 and Sunco and Tasman

3.4 Discussion

A large amount of biochemical testing was carried out to produce the raw data for PPO activity levels. With any laboratory test there will always be a certain degree of outliers produced, so it is very important to take this into account when the raw data is analysed (Bhar and Gupta 2001). The amount of outliers removed from the raw data was a relatively small 6.48%. Outliers can occur throughout this process and are usually due to experimental error, for example bubbles in the substrate, or the seed being slightly damaged. Researchers McCaig *et al.* (1999) found there was a large seed-to-seed variation when using L-tyrosine as a substrate for the PPO assay. Seed coat damage allows for more PPO to be extracted during the assay compared to undamaged and smooth seed coats.

For a number of genotypes PPO activities were re-analysed because they were showing a PPO activity level too high or too low compared to the other duplicates of that particular genotype group. To check whether this was a reproducible result, or due to experimental error, they were re-tested. Because the seeds used in this assay are subjected to a chemical substrate those exact seeds cannot be used again. Therefore for re-testing, new seed has to be

selected from seed bulked from that particular wheat line. So this, more correctly, could be termed re-selection and re-testing. Only 6 of the 36 lines that were re-tested didn't show an improved standard deviation (≤ 0.05). All of these lines were from the populations with Tasman as the Australian parent, three lines from each. The lines that did not show an improved standard deviation could still be undergoing genetic segregation, since a level of heterozygosity is still likely to be present (discussed in Chapter 2).

The descriptive information generated on the PPO activity data allows us to see the differences between the Australian and Chinese parents. These differences have enabled a number of lines to be generated, producing a lower PPO activity than any of the four parental varieties. This shows that an improvement in colour stability has been achieved. The marker haplotypes of these lines need to be tested to discover whether the desirable QTLs from the Australian parents have been transferred (Chapter 4). He *et al.* (2004) has stated that more research needs to be directed into increasing white noodle quality. The improvement in colour stability seen within these four backcross populations is an important step in this process. Habernnicht *et al.* (2002) has also found that cultivars that had relatively low PPO activity levels produced a high level of colour stability and noodle brightness (L*) in fresh noodles.

The frequency distributions of PPO activity show a substantial degree of skewness to high PPO activity in all but the Sunco x Mianyang 11 population. This skewness may be indicating additive/epistatic effects between genes regulating PPO levels in each parent and suggests that these PPO regulating loci are different in each parent. To confirm this further genetic information needs to be generated on the Chinese varieties. However, this genetic analysis is outside the scope of this current project.

It was an aim of this project to assess the possibility of improving quality attributes of the Chinese varieties by the introgression of QTLs from Australian varieties. The marker selection applied throughout the development of the four populations has been successful in producing lines that have PPO activity levels significantly different from the original Chinese parent. In the populations where the Tasman allele was selected, the majority of the transgressive segregants were directed towards an increased level of PPO activity. In the Sunco populations there were a number of lines found to have a lower PPO activity level than Sunco and either of the Chinese parents. Bonnett *et al.* (2005) also has success in enriching a population with the selection of the target alleles through the backcross breeding method. A further investigation into the genotype of these transgressive segregants has been carried out in Chapter 4.

When looking at the Xanthophyll content results a number of lines had to be re-tested because of the original result having a high standard deviation. The majority of repeat tests produced a lowered standard deviation (≤0.05) in both the comparison with the individual line (other duplicates) and when grouped with its genotype group. Overall, the re-testing of lines was successful in producing reliable data for Xanthophyll contents in all but the Tasman x Mianyang 11 population.

The frequency distributions display an obvious skew towards high Xanthophyll content in the Tasman x Chuanmai 22 and Tasman x Mianyang 11 populations. This suggests interaction between several loci may be required to produce low xanthophyll content whereas the degree of skewness in the Sunco populations is minimal. To explore the genetic implications of these distributions requires further genetic analysis of the Chinese varieties.

As low levels of PPO activity are important to Chinese white noodle production, flour and noodle brightness is influenced by Xanthophylls and other yellow pigment types (Mares and Campbell 2001, Zhang *et al.* 2008). The introgression of QTLs for both quality traits (PPO activity and Xanthophyll content) into the Chinese genetic background will then produce wheat lines

with high noodle quality attributes adapted to the growing environment in China.

The assessment of Xanthophyll content and PPO activity are time consuming assays, due to the multiple steps required for each test, and the number of tests a single operator can complete each day. Furthermore, in the case of PPO assays damaged seed and environmental influences can give artificially high readings that can confound results.

Difficulties in phenotyping have been one of the driving forces in integrating molecular markers into breeding programs, as a tool for line selection and genotype characterization (Francia *et al.* 2005). To test the efficacy of molecular markers for selecting these particular traits, the next Chapter will investigate whether the markers for PPO activity and Xanthophyll content in the two Australian lines remain linked to these characters in BC_2F_6 produced in the BC_2F_5 field trials.

Chapter 4: Analysis of the Marker Assisted Selection in the backcross populations

4.1 Introduction

Marker assisted selection (MAS) has been reported as the next step after identifying and validating QTLs found through comprehensive linkage analysis between genotyping and phenotyping data. MAS is a technique by which molecular markers are used to select lines from large populations containing genomic regions important to particular traits. Using MAS to assist the conventional backcrossing method has allowed genes/QTLs to be transferred into elite cultivars (Holland 2004). Francia *et al.* (2005) explains the main aims of MAS as; a) being able to trace favourable alleles (dominant or recessive) across generations; b) to identify the most suitable individual lines among segregating populations; and c) to break linkages with unfavourable alleles. It has been found that applying MAS at early stages of breeding allows large number of lines with unfavourable genotypes to be removed, thus reducing the number of lines to be tested (Bonnett *et al.* 2005, Liu *et al.* 2004). Using molecular markers at early generations can also reduce the number of lines which have a recombination between the marker loci and the gene/QTL. Recombination events as well as undesirable alleles at neighbouring loci linked

to the transferred gene/QTL can use cause complications with these lines in later generations (Bonnett *et al.* 2005, Eathington *et al.* 1997, Ribaut and Betrán 1999). Linkage drag is the term given to unfavourable alleles transferred with the target donor genomic region during backcrossing. When marker-assisted backcrossing is carried out, the use of tightly linked flanking markers can considerably reduce the amount of linkage drag (Stam and Zeven 1981). MAS carried out with tightly linked markers is also important in determining the number of progeny required in each backcross generation. The number of progeny required for selection for particular traits is determined by the distance between the flanking markers and the number of QTL being considered at the one time (Chao and Ukai 2000, Edwards and Page 1994). Some researchers state the distance between the flanking markers used in MAS should be 5cM or less (Chao and Ukai 2000, Edwards and Page 1994), whereas others deem for practical reasons a distance between 10-20cM is more appropriate (Visscher *et al.* 1996). For MAS to have the largest impact in selecting lines with the target genomic regions into elite cultivars, the closer the distance between the flanking markers is to zero, the greater chance of success (Hospital and Charcosset 1997).

Since the onset of this research project, it has become important to use markers to select both for the donor/target gene/QTL (termed forward selection) and also to select for the recurrent parents DNA (termed background selection). It has been reported that using MAS can accelerate the recovery of the recurrent parent genome within the Marker-assisted backcrossing method (Frisch *et al.* 1999, Frisch and Melchinger 2001). The use of background selection also reduces the number of generations required to recover most of the recurrent parent genome (see Chapter 1).

MAS has been of the most use in the assisting of backcrossing for major genes with large effects on traits with a relatively simple inheritance (Holland 2004). When more complex polygenic traits are being considered, limitations of MAS can occur. To use QTL material in MAS it is important that great care has been taken when identifying the QTLs and their position and effects, especially when considering those with smaller effects (Francia *et al.* 2005). Because QTLs are not necessarily the gene controlling a particular trait, it is very important to use two tightly linked flanking markers, severely reducing the chances of recombination between the target gene and both the flanking markers.

It is necessary to test the effectiveness and robustness of MAS before it can be an integrated method in wheat breeding and variety development. The work carried out in this Chapter assesses the usefulness of the molecular markers for successfully selecting lines with the introgressed target alleles. This analysis will indicate the ability of molecular markers to be used as predictive tools for particular phenotypes.

4.2 Materials and Methods

The genotypic data was generated from the field trial conducted in 2005 (for genotyping details see Chapter 2) and was entered into two different software packages; these being SPSS (version 15) and Map Manager QTXb20 (Manly *et al.* 2001). For the purpose of the statistical analysis, the small proportion of heterozygotes present in the four populations was removed from the genotypic data set.

The phenotypic data used for this analysis was collected through the biochemical testing of PPO activity and Xanthophyll content (see Chapter 3).

Linear regressions were carried out to compare the genotype with the phenotype (biochemical testing for PPO activity and Xanthophyll content), at a probability of 0.05. Each population was assessed individually.

Single marker regressions were carried out to analyse Xanthophyll content considering the SSR markers: wmc17, wmc346, gwm566, gwm285 and gwm108 (for Tasman x Chuanmai 22 and Tasman x Mianyang 11 populations only). The regression analysis was carried out with the PPO activity data using the SSR markers: gwm372, gwm526, wmc18, gwm157, cfd233 and gwm301 for the Sunco x Chuanmai 22 and the Sunco x Mianyang 11 populations. The SSR markers: gwm372, wmc170, gwm526, gwm102, wmc18, wmc181, gwm349 and gwm301 were used on the Tasman x Chuanmai 22 and the Tasman x Mianyang 11 populations. The marker set differs between the populations because not all markers were polymorphic between all four parents.

4.3 Results

For the data to be analysed by the computer software it was required that the heterozygotes were taken out of the genotypic data set. The numbers removed from each population differed and has been presented in Table 4-1. The numbers were quite low with the highest being 3.12% (from a total of 897), therefore the overall effect on the data analysis will be limited.

Table 4-1: Proportions of heterozygote genotypic data points removed from the genotypic data collected from each of the four backcross populations for the SPSS data analysis.

Population	Total no. of genotypic data points	No. of heterozygote genotypic data points found	Proportion of heterozygotes found
Sunco x Chuanmai 22	1240	36	2.90%
Sunco x Mianyang 11	1160	33	2.84%
Tasman x Chuanmai 22	975	27	2.77%
Tasman x Mianyang 11	897	28	3.12%

The linear regressions allowed comparisons to be made between each biochemical test and genotypic set, and the information generated from these tests has been collated in Table 4-2 and Table 4-3. Firstly each marker was investigated individually to understand their usefulness as predictive tools for selection of lines for each trait. Comparisons were made between the marker data and the PPO activities for all the population data.

Table 4-2: Results from the linear Regression analysis for PPO Activity

	PPO activity	
	R^2	Significance
Sunco x Chuan Mai22		
gwm372 (2A)	0.080	0.010*
gwm526 (2A)	0.001	0.706
wmc18 (2D)	0.063	0.011*
gwm157 (2D)	0.066	0.007*
cfd233 (2D)	0.066	0.006*
gwm301 (2D)	0.049	0.024*
Sunco x Mianyang11		
gwm372 (2A)	0.082	0.007*
gwm526 (2A)	0.054	0.022*
wmc18 (2D)	0.001	0.787
gwm157 (2D)	0.000	0.850
cfd233 (2D)	0.000	0.849
gwm301 (2D)	0.005	0.483
Tasman x Chuanmai 22		
gwm372 (2A)	0.001	0.788
wmc170 (2A)	0.291	0.000*
gwm526 (2A)	0.007	0.495
gwm102 (2D)	0.025	0.220
wmc18 (2D)	0.035	0.154
wmc181 (2D)	0.216	0.000*
gwm349 (2D)	0.143	0.001*
gwm301 (2D)	0.076	0.029*
Tasman x Mianyang 11		
gwm372 (2A)	x	x
wmc170 (2A)	0.001	0.786
gwm526 (2A)	x	x
gwm102 (2D)	0.012	0.403
wmc18 (2D	0.106	0.033*
wmc181 (2D)	0.196	0.000*
gwm349 (2D)	0.007	0.495
gwm301 (2D)	0.014	0.389

* - significance level P<0.05

X – Indicates where there was no differences found between the genotypic data, therefore no regression result can be calculated.

The chromosomal locations being investigated are the QTLs from 2A and 2D, originally identified with the Sunco x Tasman population PPO data. When considering the 2A QTL, the marker: gwm372 was found to be highly significant within both Sunco populations and explaining 8% (Sunco x Chuanmai 22 population) and 8.2% (Sunco x Mianyang 11 population) of the variation. The second marker: gwm526 only showed a significant result in the Sunco x Mianyang 11 population explaining 5.4% of the variation. These markers were not significantly linked to the trait in the Tasman populations, whereas the marker: wmc170 on chromosome 2A was found to be highly significant in the Tasman x Chuanmai 22 population only, explaining a substantial 29.1% of the variation.

When considering the 2D QTL, the four markers used all produced significant results in the Sunco x Chuanmai 22 population and two out of the four in the Sunco x Mianyang 11 population. Microsatellite marker wmc18, gwm157, cfd233 and gwm301 explained 6.3%, 6.6%, 6.6% and 4.9% of the variation respectively in the Sunco x Chuanmai 22 population. This level of significance was not found in the Sunco x Mianyang 11 population. When looking at the Tasman populations, microsatellite wmc181 proved highly significant in both populations explaining 21.6% (Tasman x Chuanmai 22) and 19.6% (Tasman x

Mianyang 11) of the variation. Similarly gwm349 explained 14.3% of the variation, but only in the Tasman x Chuanmai 22 population and wmc18 in the Tasman x Mianyang 11 population explaining 10.6% of the population.

Table 4-3: Results from the linear Regression analysis for Xanthophyll Content

	Xanthophyll Content	
	R^2	Significance
Sunco x Chuanmai 22		
wmc17 (7A)	0.108	0.002*
wmc346 (7A)	0.038	0.053
gwm566 (3B)	0.127	0.001*
gwm285 (3B)	0.135	0.000*
Sunco x Mianyang 11		
wmc17 (7A)	0.014	0.262
wmc346 (7A)	0.018	0.193
gwm566 (3B)	0.120	0.000*
gwm285 (3B)	0.158	0.000*
Tasman x Chuanmai 22		
wmc17 (7A)	0.028	0.195
wmc346 (7A)	0.344	0.000*
gwm566 (3B)	0.170	0.002*
gwm285 (3B)	0.094	0.012*
gwm108 (3B)	0.104	0.020*
Tasman x Mianyang 11		
wmc17 (7A)	0.003	0.686
wmc346 (7A)	0.107	0.016*
gwm566 (3B)	0.016	0.388
gwm285 (3B)	0.065	0.059
gwm108 (3B)	0.023	0.302

* - significance level $P<0.05$

Linear regression analysis was carried out on the Xanthophyll content data in the same manner as the PPO activity data. QTLs located on 3B and 7A were identified in the original Sunco x Tasman population and it is the microsatellites from these chromosomal areas that were used in the comparisons between the genotypic data and the Xanthophyll content phenotypic data. The 3B microsatellite markers (gwm566, gwm285 and gwm108) were found to produce significant results in three of the four populations explaining a range of the variation from 9.4% to 17% (Sunco x Chuanmai 22, Sunco x Mianyang 11 and Tasman x Chuanmai 22 populations). The microsatellite: gwm285 explained the highest amount of variation in the Sunco populations, and it was gwm566 that produced the best results in the Tasman x Chuanmai 22 population explaining the highest amount of variation for this QTL at 17%. When looking at the 7A microsatellites, wmc346 produced significant results in the Tasman x Chuanmai 22 and Tasman x Mianyang 11 populations, explaining 34.4% and 10.7% of the variation respectively. The second marker used in the genotyping was wmc17 and it was found to produce a significant result in the Sunco x Chuanmai 22 population only, explaining 10.8% of the variation. Neither of the 7A microsatellites produced significant results in the, Sunco x Mianyang 11 population.

Figure 4-1: Chromosomes 2A, 2D, 3B and 7A of the Sunco x Tasman linkage map, displaying the map distances (cM) between the microsatellites used in the genotyping of the four backcrossed populations (’03-’05)
Only SSR markers were used in this figure, the complete QTL peaks can be found in Chapter 2

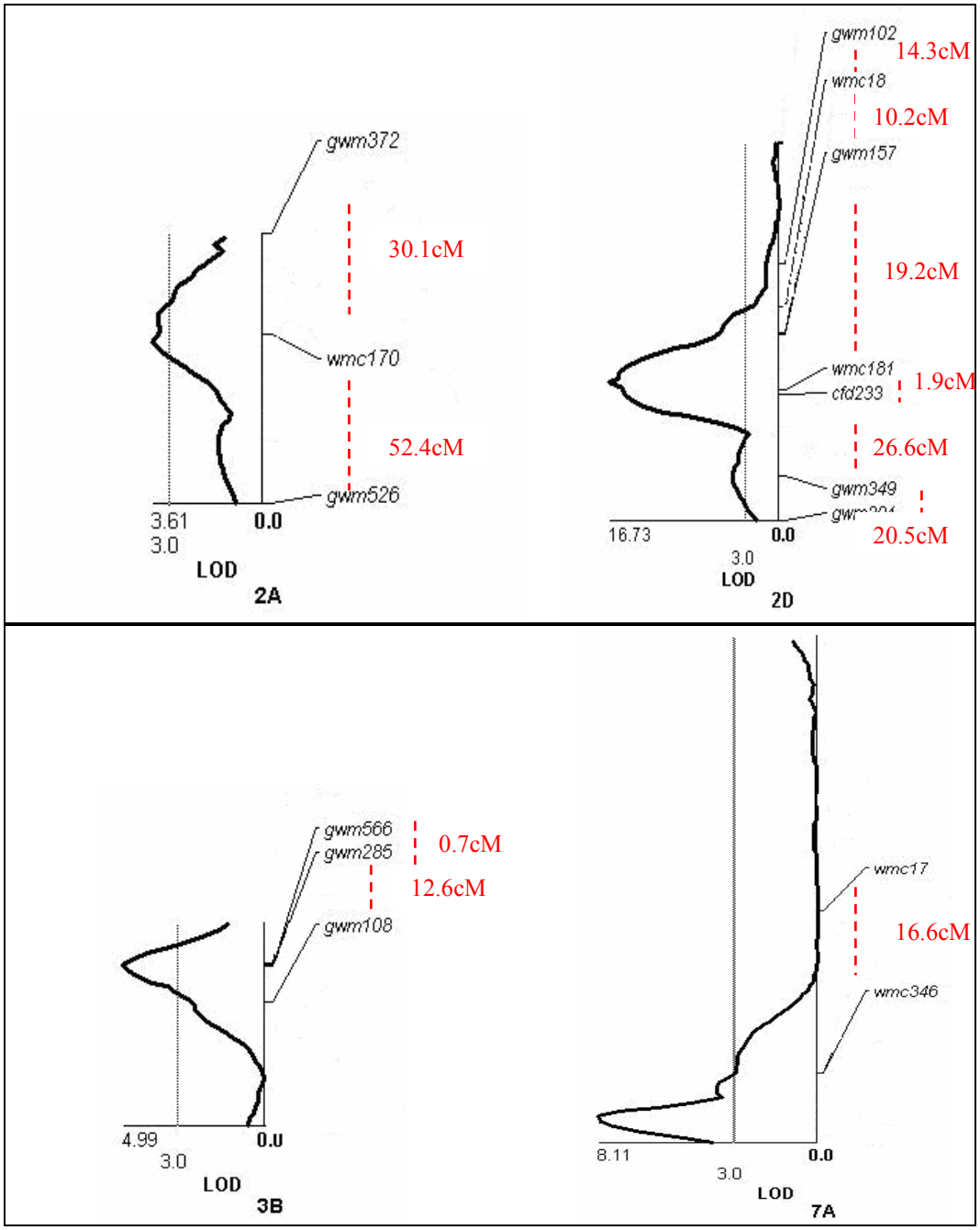


Figure 4-1 displays the map distances in cM (centamorgans) between the molecular markers used in the genotyping of the four populations (years 2003 – 2005) with the corresponding QTL traces from the Sunco x Tasman Doubled Haploid linkage map. This diagram allows for a visual assessment of the distances between the microsatellite markers, their distances away from the peak and their linkage to the traits to establish which markers should be used for flanking markers in MAS. When considering the QTL for PPO activity on 2A, the most closely linked marker is wmc170 directly under the peak, but the distances from the other markers used is quite large at 30.1cM and 52.4cM. The 2D QTL for PPO activity shows the markers wmc181 and cfd233 situated under the peak, and at a distance from 1.9cM from each other. Other microsatellites of interest in this area are gwm157 and gwm349 which are 19.2cM and 26.6cM (respectively) from the peak. Therefore these four markers are all possibilities for use in selection of lines polymorphic for these markers. When looking at the QTLs for Xanthophyll content in the Sunco x Tasman population, there were a smaller number of molecular markers to be assessed, with only three markers available under the 3B QTL and two microsatellites available under the 7A QTL. Microsatellites gwm566 and gwm285 are located directly under the peak of the 3B QTL and are only 0.7cM apart, while gwm108 is only 12.6cM proximal from the peak. Therefore either marker from

the peak and gwm108 can be used as flanking markers for this QTL. The distance between wmc17 and wmc346 from the 7A QTL is small at 16.6cM, but wmc17 is quite a distance away from the peak of the QTL.

The in-depth data analysis of all phenotypic and genotypic data has identified three lines which have produced very good results for both PPO activity and Xanthophyll content. These three lines also have alleles present for the markers found to be significant in the earlier linear regressions. These lines were generated from the Sunco x Chuanmai 22 and Sunco x Mianyang 11 backcross populations (Table 4-5).

Table 4-5: Three lines that produce a low result for PPO activity and Xanthophyll content. These lines also have marker alleles contributed by Sunco, some of which are found to be significant and able to explain a proportion of the variation (in its population). The lines below are all performing better than either Chinese variety (Chuanmai 22 and Mianyang 11)

Line	Parental Material	PPO Activity (OD415nm)	Xanthophyll Content (OD436nm)	Sunco Marker Alleles present from chromosomes 2A,2D, 3B,7A
SM65-10 (6-15-2)	Sunco Mianyang 11	0.117	0.043	2A: - 2D: gwm157 [a] , cfd233 [a] 3B: gwm285 [a] 7A: wmc17
SM57-14 (10-29-3)	Sunco Mianyang 11	0.127	0.040	2A: gwm526 [a] 2D: - 3B: gwm566 [a], gwm285 [a] 7A: -
SC33-6 (7-16-2)	Sunco Chuanmai 22	0.143	0.037	2A: - 2D: cfd233 [a] 3B: gwm285 [a] 7A: -

[a] – *Markers found to be significant in the linear regression analysis*

4.4 Discussion

Ideally this type of analysis would be carried out when all lines had finished segregating, but for the time scale of this project the field trial had to be carried out in 2005 with a small proportion of the lines in each backcross population still undergoing segregation. The numbers of heterozygotes were small (a maximum of 3.12%). At a number of generations, lines (heterozygote or homozygous for the Australian marker alleles) from these populations have been selected by molecular markers. Therefore the populations have been biased towards containing a large number of heterozygotes. If the populations had undergone random recombination through the two backcrosses and three single seed descents, then 1.56% of the total population should have been heterozygotes.

In the original research carried out on the Sunco x Tasman population by Mares and Campbell (2001) the two QTLs identified for Xanthophyll content were contributed by different parents; 3B contributed by Sunco and 7A contributed by Tasman. Two QTLs were identified for high PPO activity on 2A and 2D and they were contributed by Tasman. Tasman has a very high level of PPO activity, therefore selecting for the Sunco allele is selecting for a low PPO

activity, which is desired in the end-use products. Therefore we need to look at the four backcross populations considering the two Australian parents will be contributing very different levels of PPO activity. Ideally for the generation of lines with low PPO activities we wouldn't select Tasman as a parent, with its high level of PPO activity, but for this study, it was interesting to follow the Tasman allele to observe its effect in a Chinese background which gave a relatively low PPO activity.

Some of the molecular markers used throughout this process particularly in the 2002 selections, were more loosely linked to the four QTLs than what is ideal. This study would have benefited from using the markers used in the 2003 to 2005 genotyping from the beginning (see Chapter 2), had they been identified at that time. However the use of co-dominant microsatellite markers throughout the process was essential in identifying heterozygote and homozygous lines containing the Australian parent alleles.

An important aim of this project was to assess the usefulness of molecular markers for predicting the phenotype of a particular trait of interest. To assess this tool, comparisons were made between the genotypic and phenotypic data from the materials selected from the 2005 field trial to get an indication of

predictability of these markers for their particular trait of interest. The populations Sunco x Chuanmai 22 and Tasman x Chuanmai 22 gave the most consistent results, with significant linkage results being produced for all four of the QTLs selected. The amount of phenotypic variation explained was 34.4% (wmc346) for Xanthophyll content and 29.1% (wmc170) for PPO activity in the Tasman x Chuanmai 22 population. In the Sunco x Tasman population, the microsatellites, wmc346 and wmc170, explained a smaller amount of the phenotypic variation, this being 7% (wmc346-3B) and 11% (wmc170-2A) (Mares and Campbell 2001). The markers that explained the highest proportion of phenotypic variation in all of the four QTLs investigated was wmc181 (36%) and cfd233 (38%) for the 2D PPO QTL and gwm566 (14%) and gwm285 (11%) for the 3B Xanthophyll QTL (Mares and Campbell 2001). These markers, originally identified in the Sunco x Tasman population, were also successfully validated in the four backcross populations used in this project.

From the regression results from the PPO activity data, the microsatellite gwm372 from chromosome 2A is effective in selecting for low PPO activity in both Sunco x Chuanmai 22 and Sunco x Mianyang 11 populations. Microsatellite wmc170 produced very significant results in the Tasman x Chuanmai 22 population, and is located at the peak of the 2A QTL. The 2A

QTL was contributed by Tasman in the Sunco x Tasman population; therefore using the Tasman allele for this marker should confer a high PPO activity level. Unfortunately this marker could not be used to select for the Sunco allele (selecting against a high PPO activity level) in the Sunco populations because it was not polymorphic between Sunco and the Chinese varieties. Chromosome 2D has a number of microsatellites that produced significant results and could be used as flanking markers. In Figure 4-1, wmc181, cfd233 are the two microsatellites situated at the most ideal position, directly under the QTL peak. The microsatellites gwm349 and gwm157 are located further away from the peak, but still close enough to produce very significant results in the regression data analysis and would be very useful as alternatives in populations where the closer SSRs were not polymorphic. Used in conjunction with wmc181 and cfd233 they would be useful in backcrosses to select lines with lower linkage drag. Lines containing linkage drag can occur when using more distant flanking markers and with the markers directly under the QTL peak.

Markers on chromosome 3B showed significant linkage to lower Xanthophyll content. Microsatellites gwm285 and gwm566 were linked to Xanthophyll content variation in three of the populations (Sunco x Chuanmai 22; Sunco x Mianyang 11; Tasman x Chuanmai 22). Another promising aspect of these

markers is that they are tightly linked at 0.7cM apart on the chromosome, making them ideal to be used as flanking markers. In contrast the results for markers on chromosome 7A failed to demonstrate a significant linkage with Xanthophyll content. This may be due to the 7A markers used during the selection process for Xanthophyll content. In the Sunco x Tasman population these markers only explained a small proportion of the phenotypic variation, at 7% for wmc346 and 0% for wmc17. These markers were the only microsatellite markers available under the 7A QTL; therefore they were included in the selection process. To successful introgress this QTL into different genetic backgrounds, markers more tightly linked to the Xanthophyll content trait are needed.

For MAS to be successful it is essential for the markers being used to be as tightly linked to the QTL as possible and for the map distances between flanking markers to be as small as possible. The robustness of the markers was increased by the work of Lehmensiek *et al.* (2005), but this occurred after the first round of selections were made in 2002 (see Chapter 2). There may have been unwanted segments of chromosomes being transferred via linkage drag and lines carrying the QTL may have been discarded during selection due to loss of linkage to the markers following recombination (Holland 2004). These

problems are also occurring when the flanking markers used in MAS are too far apart from each other and/or distant from the peak of the QTL. The literature supports the use of flanking markers at a distance of 5-20cM apart (Chao and Ukai 2000, Edwards and Page 1994, Visscher *et al.* 1996).

Another aim of this project was to successfully introgress one or more QTLs (2A, 2D: PPO activity; 3B, 7A: Xanthophyll Content) from an Australian variety into the Chinese genetic background. Considering all of the analysis carried out in this chapter, it is evident that the introgressions could have been carried out more successfully with different or more molecular markers, but there were four lines that were highlighted as important and worth further investigation. These lines contained differing combinations of the QTLs, with lines containing three of four QTLs (Line A: 2D, 3B and 7A; Line D: 2A, 2D and 7A) and lines containing two of the four QTLs (Line B: 2A and 3B; Line C: 2D and 3B). These lines can now be tested further in Australia and China to ensure that the selection made from these molecular markers has conferred a beneficial phenotype in both the Australian and Chinese environments.

Chapter 5: General Discussion

5.1 Research Outcomes and the Impact of this Project on Wheat Improvement

The project objectives outlined in the rationale for the study (Chapter 1) were all met throughout this research into Marker-assisted selection and its impact on transferring Quantitative Trait Loci from Australian wheats into Chinese wheats.

The objectives of this study were:

1. To use the QTLs previously identified by Mares and Campbell (2001) to improve the flour colour characteristics in backcross populations generated between Australian varieties (Sunco and Tasman) and Chinese varieties (Chuanmai 22 and Mianyang 11).

2. To evaluate Marker-assisted selection as an efficient method of transferring QTLs located in Australian germplasm to a Chinese genetic background.

3. To test the usefulness of microsatellite markers as a tool to predict phenotype (Polyphenol oxidase activity and Xanthophyll content) in a novel genetic background.

4. To increase awareness and usefulness of molecular markers in wheat breeding in China.

The backcross populations generated in this project have allowed comprehensive genotypic and phenotypic data to be produced, all of which are essential in completing the aims of this project. The most important areas of this study for wheat breeding and its improvement are: the identification of microsatellites which confer a particular phenotype, and the ability to use marker-assisted selection to improve the final colour of wheat flour and its products.

This project was successful in highlighting microsatellites capable of identifying wheat lines with differing levels of PPO activity and Xanthophyll content. The microsatellites gwm372, wmc170 (2A – PPO), wmc181, cfd233 (2D – PPO) and gwm285 and gwm566 (3B – Xanthophyll) are all potential

markers for use in wheat breeding programs in Australia and China. This work has also further validated these microsatellite markers which were first proved to be useful in the Sunco x Tasman Population.

By using the above microsatellites, three wheat lines have been developed containing different combinations of the four Sunco x Tasman QTLs. These lines now have QTLs conferring low PPO activity and low Xanthophyll content, both appropriate characters to produce high quality end-products. As explained in Chapter 1, the Chinese market demands wheats that produce fresh noodles with a bright white stable colour. Therefore wheat lines produced from this project may, with further investigation, confer the good quality attributes required of wheat in China.

5.2 Future Directions

There are a number of areas resulting from this project that could benefit from continued research. These are:

a) Marker validation of the above acknowledged microsatellites needs to be carried out under Australian and Chinese environmental conditions to ensure

these markers continue to predict the necessary phenotype. This current project was only carried out in Australia and over one growing season. Willcox *et al.* (2002) reports that a single season's data is insufficient in the identification of QTLs, therefore it is important to test the markers found linked to QTLs over a number of years also.

b) A more comprehensive study is required to assess the potential of the four lines generated from this project. They have been shown to contain introgressed QTL material from Sunco, but further phenotypic data on flour and noodle colour should be produced to test these genomic areas are conferring the appropriate end-product quality in a range of environments.

c) A number of studies have used a method termed "background marker selection" to enhance the recovery of the recurrent parent genotype during backcrossing, and to limit the amount of linkage drag occurring during the QTL introgression (Bonnett *et al.* 2005, Howes *et al.* 1998, Kuchel *et al.* 2007). This type of marker selection was not carried out during this project therefore it is not known what effect linkage drag has had on the lines developed. To better understand the amount of genomic material surrounding the QTLs transferred,

markers could be screened across the genome to assess the amount of recurrent (Chinese) parent genotype present.

d) The markers available from the 7A QTL were not able to explain the variation in Xanthophyll content in this project. If this QTL is to be studied further an effort to identify other markers on 7A is required.

5.3 Conclusion

Markers have been used successfully in wheat breeding programs around the world. However, for markers to become a completely integrated tool, they must be reliable and efficient at all times. This project has highlighted the usefulness of molecular markers in the improvement of quality attributes (colour) in wheat varieties.

This project has established links between Australia and China, and this collaboration enables the sharing of elite germplasm, phenotypic and genotypic techniques and the potential to improve the current varieties available to farmers.

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Appendix A: Protocols used in the Genotypic and Phenotypic assessment of the backcross populations

Protocol 1:

Phenol/Chloroform Method for genomic DNA Extraction

1. Leaf material ground using liquid nitrogen in a mortar and pestle until a fine white powder was formed.
2. 4ml of *Extraction Buffer* added and mixed.
3. 4ml of *Phenol/Chloroform/Iso-amylalcohol* (25:24:1) added and mixed.
4. Centrifuge samples at 5000rpm for 10 minutes in a bench-top centrifuge.
5. Top Phase Layer of the supernatant transferred into a clean tube
6. 400ml of *Phenol/Chloroform/Iso-amylalcohol* (25:24:1) added for a re-extraction step.
7. Centrifuge samples at 5000rpm for 10 minutes.
8. Top Phase Layer of the supernatant again transferred into a clean tube
9. 400µl *3M Sodium Acetate* (pH 4.8) and 4 ml Isopropanol. Mixed very gently
10. Centrifuge samples at 5000rpm for 10 minutes to pellet DNA.
11. Gently remove and discard supernatant. Wash DNA pellet with 4ml *70% Ethanol*.
12. Centrifuge at 5000rpm for 5 minutes.
13. Remove and discard supernatant and allow DNA pellet to air dry.
14. Add 350µl *1x TE Buffer* to dried DNA pellet for resuspending the DNA.
15. Store DNA samples at 4^{o}C for short term used or store at -20^{o}C for long term storage.

Reagents:

DNA Extraction Buffer	TE Buffer
1% Sarkosyl	10mM Tris-HCl
100mM Tris-HCl	1mM EDTA
100mM NaCl	pH 8.0
10mM EDTA	
pH 8.5	

Protocol 2:

Wizard Genomic DNA Extraction (tubes)

Reagents for this method are commercially made therefore the exact components are not known.

1. Into a 1.5ml microfuge tube place a sample of leaf material, add 4 ball bearings
2. Add **600µl** of *Nuclei Lysis Solution*
3. Place the tubes into the Mixer Mill Adapters and mix for 2 mins at 30 shakes/sec.
   Repeat.
4. Incubate at 65^{o}C for 15 mins
5. Add **10-12µl** of *RNase Solution* to the lysate, Mix (by inverting tubes 2-5 times)
   Incubate at 37^{o}C for 15 mins
   Allow the sample to cool to Room Temp for 5 mins before proceeding

6. Add **200µl** of *Protein Precipitation Solution* and Vortex vigorously at high speed for

20 secs

7. Centrifuge for 3 mins at 13 000 – 16 000 x g (14,000 x g = 11, 481rpm)

Precipitated proteins will form a tight pellet

8. Carefully remove the supernatant containing the DNA

Transfer into a clean 1.5ml microfuge tube containing **600 µl** *Isopropanol* (Room Temp).

9. Gently Mix the solution by inversion until thread-like strands of DNA form a visible mass.

10. Centrifuge at 13 000 – 16 000 x g (14,000 x g = 11, 481rpm) for 1 min at Room Temp

11. Carefully remove the supernatant

Add **600µl** *70% Ethanol* (Room Temp) and gently invert the tube several times to wash

the DNA pellet.

12. Centrifuge at 13 000 – 16 000 x g (14,000 x g = 11, 481rpm) for 1 min at Room Temp.

13. Carefully aspirate the ethanol using a pasteur pipette

(DNA pellet is very loose at this point)

14. Invert the tube and air-dry the pellet for 15 mins

15. Add **100µl** of *DNA Rehydration Solution or sterile milli Q water* and re-hydrate the DNA by incubating at 65 ^{o}C for 1 hr, periodically mixing.

Or, incubation the solution overnight at Room Temp or 4^{o}C.

16. Store DNA at 4^{o}C

Protocol 3:

Wizard Genomic DNA Extraction (plates)

Reagents for this method are commercially made therefore the exact components are not known.

1. Into each tube of the 96 well plate place a sample of leaf material, add 4 ball bearings
2. Add **500µl** of *Nuclei Lysis Solution*
3. Place the plate into the Mixer Mill Adapters and mix for 2 mins at 30 shakes/sec.
   Repeat, changing sides.
4. Incubate at 65^{o}C for 15 mins
5. Add **10-12µl** of *RNase Solution* to the lysate, Mix (by inverting tubes 2-5 times)
   Incubate at 37 ^{o}C for 15 mins
   Allow the sample to cool to Room Temp for 5 mins before proceeding
6. Add **200µl** of *Protein Precipitation Solution* and Vortex vigorously at high speed for
   20 secs
7. Centrifuge for 3 mins at 4000 rpm, Precipitated proteins will form a tight pellet
8. Carefully remove the supernatant containing the DNA
   Transfer into a clean tubes containing **500 µl** *Isopropanol* (Room Temp).
9. Gently Mix the solution by inversion until thread-like strands of DNA form a visible mass.

10. Centrifuge at 4000rpm for 1 min at Room Temp

11. Carefully remove the supernatant

Add **500µl** *70% Ethanol* (Room Temp) and gently invert the tube several times to wash

the DNA pellet.

12. Centrifuge at 4000rpm for 1 min at Room Temp.

13. Carefully remove the ethanol (DNA pellet is very loose at this point)

14. Invert the tube and air-dry the pellet for 15 mins

15. Add **100µl** of *DNA Rehydration Solution or sterile milli Q water* and re-hydrate the DNA by

incubating at 65 ^{o}C for 1 hr, periodically mixing.

Or, incubation the solution overnight at Room Temp or 4^{o}C.

16. Store DNA at 2 – 8^{o}C

Protocol 4:

Qiagen DNeasy Plant DNA Purification Kit

All Reagents supplied with the Qiagen Kit

1. Leaf tissue ground using liquid nitrogen in a mortar and pestle until a fine white powder is produced. Transfer into a microfuge tube.

2. Add 400µl of *Buffer AP1* and 4µl of *RNase A solution* (100mg/ml). Mix well.

3. Incubate samples at 65^{o}C for 10 minutes, mixing during this time.

4. Add 130µl of *Buffer AP2* and mix. Incubate for a further 5 minutes on ice.

5. Centrifuge samples at 14,000rpm for 5 minutes.

6. Transfer the supernatant into the QIAshredder Mini spin column and centrifuge at 14,000rpm for 2 minutes.
7. Transfer the flow-through fraction into a new tube.
8. Add 1.5 volumes of *Buffer AP3/E* to the cleared lysate and mix.
9. Pipette 650µl of the mixture into the DNeasy Mini spin column. Centrifuge at 8000rpm for 1 mintue. Discard the flow-through.
10. Repeat the above step with the remaining sample and discard the flow-through.
11. Place the DNeasy Mini spin column into a new tube and add 500µl of *Buffer AW*. Centrifuge at 8000rpm for 1 mintue. Discard the flow-through.
12. Add 500µl of *Buffer AW* to the spin column and centrifuge at 14,000rpm for 2 mintues to dry the membrane.
13. Transfer the DNeasy Mini spin column to a new tube and add 100µl *Buffer AE* directly onto the membrane. Incubate for 5 minutes at Room Temperature. Centrifuge at 8000rpm for 1 minute.
14. Repeat Step 13.
15. Store the eluted DNA at 4^{o}C for general use and at -20^{o}C for long term storage

Protocol 5:

Single Seed Assay for Polyphenol oxidase Activity

Stock Solutions:

a) Tween 80 Stock Solution: 20ml Tween 80 made up to 100ml with water

b) Tyrosine Solution: 0.225g Tyrosine (di-sodium salt) and 1.21g Trizma base in 80ml water, pH 9.0. Add 10ml Tween 80 stock solution and making the total volume up to 100ml with water.

Single seeds were placed into each well of a microplate except for the first row which serves as the reagent blank. Single seeds of a particular genotype were placed in every two rows of the plate (16 wells). Last row of the PPO standard: cultivar Sunco

1. Tyrosine solution (200µl) was added to each well of the plate using a multi-channel pipette.
2. Each plate was covered and incubated for 3 hours at 37^{o}C.
3. After incubation, 100µl of the substrate was removed from each well and placed into a clean microplate.
4. Microplate was put into a microplate reader, and optical densities calculated at 415nm.