IDENTIFICATION OF MOLECULAR MARKERS IN WHEAT AND MAIZE USING AFLP TECHNOLOGY

IZAK SCHALK PETRUS VAN NIEKERK

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PREFACE

The experimental work described in this dissertation was conducted at department of Biotechnology, CSIR/Biochemtek, Pretoria, under the supervision of Dr. Eugenia Barros and Professor Annabel Fossey.

The results have not been submitted in any other form to another University and except where the work of other is acknowledged in the text, are the results of my own investigation.

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I certify the above statement is correct.

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ABSTRACT

The optimisation and implementation of a molecular fingerprinting system and its ultimate use in marker assisted selection of identified maize and wheat genotypes for the CSIR are described in this dissertation. The Amplified Fragment Length Polymorphism (AFLP) method was optimised for the maize trait *gamete sterility,* and for the wheat traits *eye spot resistance* and *cytoplasmic male fertility*.

Maize and wheat DNA were extracted from selected genotypes employing standardized protocols. A commercial AFLP kit was used to obtain suitable fingerprint fragments for analysis. As this kit was not designed for very large genomes, conditions for the large wheat genome required optimisation.

AFLP fingerprints generated from the different genotypes of maize and wheat were evaluated for potentially trait linked markers. Polymorphic fragments were identified by their presence in genotypes containing the trait of interest, and their absence from fingerprints of genotypes that did not contain the trait of interest. These amplification products were identified and selected as possible markers. These potential markers were converted to Sequence Characterised Amplified Regions (SCARs) by cloning the selected amplification products to T-tailed pSK vector, and subsequent sequencing of the fragment.

Primers were designed to recognise these amplification regions in the tested genotypes. Primers were optimally designed to include or exclude the restriction sites of the enzymes at each end of the amplified fragments and annealing

temperatures normalised for each of the primer sets. The primers were validated by amplification of the tested genotypes with the primer sets at specific reaction and temperature cycling conditions.

For maize, two primer sets were identified that could be developed to identify *gamete sterility* genotypes. In the case of wheat, one primer set for each of the traits *eye spot resistance* and *cytoplasmic male fertility* was identified for their potential use as markers.

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LITERATURE REVIEW

1.1 INTRODUCTION

Early plant breeders developed new varieties of crops without prior knowledge of the gene(s) controlling traits of interest (Tanksley *et al.,* 1995) by selecting superior plants based upon their phenotypes. These pioneer varieties have formed the foundation of modern day plant breeding practices.

Most economically important traits are not controlled by a single gene, but rather by a set of genes acting together (Falconer, 1989). Traits that are controlled by multiple genes, such as for size and weight, tend to have a more subtle, constant variation in the resultant phenotype, leading to a continuous distribution of phenotypic values. These are also known as *quantitative* traits. Traits controlled by one or a very few genes, such as certain diseases, tend to have more drastic, discrete changes in expression, and these are termed *qualitative* traits (Snustad and Simmons, 2003).

Many single gene traits have been mapped to relatively precise locations by observing the segregation of the trait in a mapping population. The genotypes of many traits, especially quantitative traits, are often influenced by the environment to a greater or lesser extent (Rafalski and Tingey, 1993). This results in the masking of a genotype and when a plant's genetic merit is assessed based only on its phenotype the measurement can be very inaccurate. Tools that will assist with more accurate assessment of genotypes will greatly facilitate genotype identification and selection in plant breeding.

In modern plant breeding the genetic improvement of crops have been greatly influenced by the application of the newer DNA-based diagnostic technologies which

have quickened genetic advancement significantly (Chrispeels and Sadava, 2003). Direct assessment of genotypes has now become an integral component of many plant breeding programmes. Finding DNA markers that are linked to phenotypic traits of importance have become totally integrated into breeding programmes. Of particular importance is the use of molecular markers to identify multigenic and quantitative traits, which have been notoriously difficult to work with in the past when breeders had to rely upon phenotypic evaluation alone.

Techniques to analyze DNA, developed within the last twenty years, are enabling breeders to locate specific DNA genes or chromosome regions that are associated with particular traits. Using these techniques, breeders can choose superior individual plants to breed more quickly and accurately, speeding the overall process of genetic improvement. This technology is mostly used to fingerprint genotypes, either to identify parents to be used in a breeding programme or to identify genotypes to be selected during the breeding process.

1.2 GENETIC MARKERS

An observable gene with simple Mendelian inheritance could act as a marker for the segregation of a gene involved in the expression of a trait. Early work on the assessment of genome variation of potential markers focused upon the analysis of proteins and in animals on blood type variation. The advent of molecular technologies has made it possible to assess genomes directly, independently of the environment.

1.2.1 Types of genetic markers

Generally, there are three types of markers used in plant breeding. Morphological markers that are based on visible traits (phenotypic expression), such as flower

colour, seed colour, seed shape etc., and secondly, biochemical markers that are based on the detection of natural enzymes (isozymes) being produced. Isozymes are often not chemically identical (allozymes), indicating allelic mutations, which can be detected by electrophoresis (Chalmers *et al.*, 1992). The third type is the group of molecular markers that are based on DNA polymorphisms detected by DNA probes or amplified products of the Polymerase Chain Reaction (PCR).

There are a number of important disadvantages that pertain specifically to morphological and biochemical markers:

1. Morphological and biochemical markers are products of gene expression; products of translation.
2. Phenotypes are determined by the genetic composition and the environment in which plants grow.
3. Effects of the environment can mask genotypic effects and the phenotype therefore provides an imperfect measure of a plant's genetic potential.
4. Developmental constraints; gene products may be restricted to specific organs or to a particular time of development.
5. Expressed genes are subjected to dominance, recessiveness and epistasis; can be polygenic (controlled by multiple genes) in nature etc.
6. Lack adequate polymorphisms.
7. Lack coverage of the genome.

Molecular markers on the other hand have a number of advantages:

1. Molecular markers are not products of translation and therefore cannot be affected by the environment.
2. Provide a true representation of the genetic make-up at DNA level.
3. There are no developmental constraints during analyses.
4. No prior knowledge of dominance / recessiveness of a particular trait are necessary.
5. Unlimited number of markers.

1.2.2 Molecular genetic markers

DNA fingerprinting, identification analysis, profiling or typing all refers to the characteriation of one or more relatively rare features of an individual's genome. Every organism is phenotypically unique, determined by its genotype and environmental influences. The exception to this is identical twins in animals and humans, and clones in animals and plants. The DNA fingerprinting technology in plants focuses mainly on intra-population studies, where there is a need to identify individuals and close relatives; population based studies, looking at population structure, size and population-specific markers (Haig, 1998); genetic map construction and identification of markers for use in plant breeding.

Extended linkage maps have been constructed for a great number of agronomic crops and annual plant species. The development of Restriction Fragment Length Polymorphisms (RFLPs) for high density genomic mapping (Botstein *et al*., 1980) provided a molecular technique which overcame some of the problems associated with isozymes and proteins. Although RFLPs are unlimited, they require elaborate laboratory techniques which make them labour intensive, time consuming and costly.

Most molecular markers are not genes in the classical sense in that they do not encode for a particular protein product. They are however extremely useful as they are constant 'landmarks' in the genome which display a large amount of polymorphism. The different polymorphic types are termed 'alleles' and it is these alleles that are the essential ingredient required for the fingerprinting process (Parker *et al.*, 1998). These markers therefore provide a means for fine-grained analysis of an individual's genotype.

The most useful techniques are those that produce a large number of alleles at a single locus, or many loci with one or more common allele (Parker *et al.*, 1998). Two routes of analyses may be pursued when trying to analyse the polymorphisms for individual identification. The first route known as the single-locus approach, aims at one locus, whereas the second, analyses several loci simultaneously and is termed

the multi-locus approach. The multi-locus approach yields a DNA fingerprint in one step, as compared to the single-locus approach, which requires the combination of several locus-specific assays to achieve the same multi-locus DNA profile (Krawczak and Schmidtke, 1994).

At present a number of techniques are available that fall under either random methods of detection or specific methods. The most important methods involved in detecting specific sites on the DNA are restriction fragment length polymorphisms (RFLPs), micro- and minisatellites. Random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) in contrast detect random sites.

Single-locus Markers

Single-locus markers are DNA sequences that occur only once per genome. They require specific primers to be amplified. Prior knowledge about the sequence of these potential markers is required for amplification.

RFLPs are based on the heritable polymorphisms in the length of fragments produced by digestion of DNA with restriction enzymes. If a restriction site is present on a strand of DNA, the DNA strand will be cleaved by the corresponding restriction enzyme that would recognise the restriction site. This would result in the strand decreasing in size and thus showing up as two different bands on the gel. Differences in the length of the fragments generated occur as a result of mutation or changes in the base sequence of the DNA through insertions, deletions and base substitutions. These changes will be carried through to the offspring of that individual providing a means for determining paternity (Snustad and Simmons, 2003).

The advantage of using RFLPs is that they are highly polymorphic, co-dominant markers, allowing one to distinguish between homozygous dominant individuals and heterozygotes. However, in order to amplify a specific region, sequence information is

required; therefore, RFLPs can only be used if sufficient sequence information is available. The technique is also expensive and time consuming.

RFLP analysis, being a single-locus marker, gives a very simple DNA profile, namely that of the locus analysed. The resultant fingerprint would therefore only contain a few fragments. However, single-locus markers can also appear as a multi-locus profile by amplifying several markers, each specific for a single locus, simultaneously. This 'cocktail' of markers will result in a multi-locus fingerprint, much the same as that obtained by random amplified markers (Krawczak and Schmidtke, 1994).

Simple sequence repeats (SSRs) exhibit variation in the number of tandem repeat sequences. There are numerous areas in genomes of organisms containing these repeats. The copy number of such core sequences reveals genetic variation or polymorphisms between individuals, where the alleles are exhibited by different numbers of repeats resulting in amplified fragments of different lengths. SSRs are termed micro- or minisatellites depending on the length of the repeat sequence. The repeat sequences of minisatellites consist of a maximum of 80 base pairs; while those of microsatellites are two and four base pairs long (Parker *et al.,* 1998). These markers have become very popular because they are abundant and display many alleles. A major drawback of these markers is that they require prior sequence information, which is often unavailable. This requires detailed research of the organism's sequence structure before the fingerprinting analysis can even be conducted. Furthermore, if multi-locus profiles are required, the procedure is extremely time-consuming as several markers each amplifying one locus need to be screened (Bruford *et al.,* 1993).

Multi-locus Markers

Multi-locus fingerprints can be generated either by the simultaneous application of several single-locus markers, each one specific for one locus, or by applying a single molecular marker that simultaneously detects several loci. The latter option is the one that is preferred by researchers as it offers several advantages over the first method

(Karp *et al.*, 1996). Using one marker, which is multi-locus in nature, is much easier and less time-consuming than having to amplify several markers simultaneously. Multi-locus fingerprints have high information content and are ideal for individual identification.

Williams *et al.* (1990) developed a genetic marker, randomly amplified polymorphic DNA (RAPD) which do not require prior knowledge about the organism's genome. RAPD analysis is based on a single primer 10 – 11 nucleotide bases in length, which anneal to multiple sites on the template DNA due to their short sequence nature (Caetano-Anollés *et al.*, 1991). If these primers anneal in the correct orientation and are a suitable distance apart, the unknown sequence between them is amplified, resulting in visualised fragments on an agarose gel. Polymorphisms between genotypes are due to either a nucleotide base change that alters the ability of the primer to anneal to the template, or an insertion or deletion within the amplified fragment. The alleles are therefore seen as the 'presence' or 'absence' of a particular RAPD product, the presence or absence of fragments on the gel (Waugh and Powell, 1992 ;Ellsworth *et al.*, 1993).

RAPDs have become very important genetic markers because the assay is simple, fast and relatively inexpensive, allowing many loci to be identified in a single reaction (Foolad *et al.*, 1993). Furthermore, only small amounts of DNA are required and no prior knowledge of the genome in question is necessary. The major disadvantage of using RAPDs is the dominant nature, which means that heterozygotes cannot be distinguished from homozygous dominant individuals' locus (Williams *et al*., 1990; Welsh *et al*., 1992). Furthermore, the results show inconsistencies which means that conditions need to be carefully standardised from run to run to obtain consistent results.

Another molecular marker that also uses the multi-locus system is the amplified fragment length polymorphism (AFLP) system (Vos *et al.,* 1995). AFLPs are very similar to RAPDs. It requires no prior sequence knowledge and is based on random

amplification and identification of polymorphisms at many DNA loci.

The AFLP technique is based on multiple amplification using PCR of a subset of restriction fragments from a total restriction digestion of genomic DNA. The DNA is cut with two enzymes, a rare and a frequent cutter according to the length of their restriction site. Each fragment is then ligated to adapters that serve as a binding site for primers. Only fragments that have both restriction sites (one at either end) are amplified by PCR. This initial amplification, pre-selective amplification, reduces the total number of restricted fragments present in the reaction. The PCR product is then used as a template for a second amplification, selective amplification, using primers with three additional selective nucleotides included at the 3' end. The amplification is selective because one selective nucleotide on each primer results in the amplification of only 1 out of 16 fragments, while two selective primers will reduce this value to 1/256. Therefore, the number of resulting amplified fragments is determined by the number and composition of the selective nucleotides used. The final product of the whole process is a multi-locus fingerprint-like pattern on a gel that can be scored with a manually or by an automated sequencer (Blears *et al.*, 1998).

The advantage of using AFLPs is that many fragments (approximately 50-100) are identified, thereby displaying multiple visible polymorphisms. Only a small amount of DNA is needed and no probe hybridisation is required. The fingerprint also seems to be more consistent than those found in RAPDs. The major disadvantages of this method, however are that it is significantly more expensive than RAPD analysis and also technically challenging. Furthermore, like RAPDs, AFLPs cannot distinguish between heterozygous and homozygous dominant individuals as the process is based on the 'presence' and 'absence' of fragments (Pejic *et al.*, 1998). AFLPs have greatly changed the prospects for application of molecular markers to study populations and to accelerate breeding (Rafalski *et al.,* 1991; Rafalski and Tingey, 1993). These markers provide a very powerful tool to generate relatively dense linkage maps in a short period of time.

An important application of RAPDs or AFLPs is to sequence the polymorphic fragments and convert these possible markers to SCARs (Paran and Michelmore, 1993), sequenced characterized amplified regions. While SCARs will allow for rapid development of sequence tagged sites (STS), they have the potential to be very useful in marker assisted selection and have been developed for many different crops (Williams *et al*., 1991a; Tragoonrung *et al*., 1992). A useful STS (Olson *et al*., 1989) is a unique, simple-copy segment of the genome which DNA sequence is known and which can be amplified by a specific PCR.

1.3 MOLECULAR MARKERS IN PLANT BREEDING

The identification of molecular markers is very useful in plant breeding. They assist breeders in determining the location of genes that control important traits, referred to as marker-assisted selection (MAS). In MAS, a piece of DNA is associated with a desirable trait and its presence is used to select plants that have that piece of DNA. It is often easier and less expensive to select plants that have the DNA marker than it is to grow them to maturity and see if they have developed the desired trait.

Many programmes have been launched to characterise the genomes of economically important species. The goal of these programmes is to locate markers at regular intervals along the DNA of all the chromosomes, so that virtually any observable trait can be traced to a specific stretch of DNA by routine laboratory procedures. The distance between a marker and the DNA of interest is very important. If a marker is very close to the stretch of DNA of interest (closest association would be inside the gene if interest), the probability of breaking this association through crossing-over is small (Snustad and Simmons, 2003). The greater this distance, the greater the potential that crossing-over can break the association. It is therefore important to establish to which allele of a DNA segment a marker is linked, before it is used in a particular analysis / ‘family’ of gentoypes.

Genetic linkage maps exist for many plant species, including domesticated crops such as barley and rice. In barley, there are well-established genetic linkage maps generated by RFLPs, RAPDs (Kleinhofs *et al.*, 1993), SSRs (Becker and Heun, 1995) and AFLPs (Castiglioni *et al.*, 1998). These maps on their own cover only a portion of the genome; combined maps were produced by Becker *et al.* (1995), Liu *et al.* (1996) and Powell *et al.* (1997) that covered a greater portion of the genome. Using linkage maps of barley, Heun *et al.* (1991) placed two known genes, *hulless* and *Mla12* powdery mildew resistance among the assigned markers and linkage groups. Kretschmer *et al.* (1997) linked the *Ha2* cereal cyst nematode resistance gene to two RFLP probes on the 2HL arm of barley after screening F_2 progeny with RFLP probes linked to the specific chromosome arm.

Genetic maps for rice generated from RFLP (Causse *et al.*, 1994; Jena *et al.*, 1994) and SSR (Panaud *et al.*, 1996; McCouch *et al.*, 1997) are also well established. For further enrichment of the genetic maps, Panaud *et al.* (1996) and Maheswaran *et al.* (1997) integrated SSR and AFLP maps into existing RFLP maps of rice. Using a mapping population, Wang *et al.* (1994a) identified two dominant RFLP loci with qualitative resistance to *blast* disease in rice with five species of the disease causing fungus. Genetic maps for crops such as sorghum (Hulbert *et al.*, 1990), oat (O'Donoughue *et al.*, 1995) and soybean (Keim *et al.*, 1997) have been used in the identification of quantitative trait loci (QTL). QTLs describing developmental intervals (grain filling period, days from planting to heading) and number of plants, could be identified in oat by a subset of RFLPs that showed great association between the markers identified in linkage maps and the traits (Beer *et al.*, 1997). In potato, AFLP fingerprinting was used to map the *Rx* gene for extreme resistance to potato virus X (Bendahmane *et al.*, 1997).

In some breeding programmes the objective is not necessary the mapping of a trait, but to obtain the physical linkage of a marker to the trait. Kutcher *et al*. (1996) identified a RAPD marker in barley that identified *root spot blotch* disease. Complete resistance to an isolate of the *blast* fungus was identified by two RAPD markers (Naqvi and Chatoo, 1996) that are tightly linked to a dominant *blast-resistance* gene

(*Pi-10*). In pearl millet (*Pennisetium squamulatum*) RAPD markers tightly linked to apomixes are used to identify aposporous individuals in a genetic cross (Ozias-Akins *et al.*, 1998). The *root-knot nematode resistance* locus *Mi* in tomato was identified by analysis of a F_2 population with AFLP markers in cosmid subclones (Kaloshian *et al.*, 1998). Linkage was established between AFLP markers and disease resistance against *Melampsora larici* – populina in *Populus* species (Cervera *et al.*, 1996a; 1996b).

1.4 CROPS IN SOUTH AFRICA

The grass family (*Poaceae*) with approximately 10,000 species and 660 genera is the most ecologically and economically important of all plant families. The important cereals such as wheat, rice, maize, barley, oat and rye all belong to this family and were most likely amongst the first plants domesticated by early civilizations (Table 1.1) (Arumuganathan and Earle, 1991; Bennetzen and Freeling, 1993; Bennet and Leitch, 1995). These crops have exerted important influences on the development and economy of human civilizations and even today they constitute the most important group of food consumed. The percentage contribution of these grains to the national gross income is an indication of their importance in South Africa. Both maize and wheat contribute the largest percentage income of crop plants in South Africa with maize responsible for about 34% of the total income of field crops and contributing 10% to the income of the total agricultural sector. Wheat, on the other hand, contributed 14% of the total field crop income and 4,5% to the total agricultural income.

Table 1.1 Summary of general characteristics of the main grass species (Table compiled from Arumuganathan and Earle, 1991; Bennetzen and Freeling, 1993; Bennet and Leitch, 1995).

Species	Crop	Origin	Time of origin	Use	Reproduction	Ploidy
Avena strigosa-brevis	oat	Iberia	0-5000BC	fodder	ib/sd	2x
A. abyssinica	oat	Ethiopia	0-5000BC	cereal	ib/sd	auto 4x
A. sativa-byzantina	oat	Europe	0-5000BC	cereal	ib/sd	allo 6x
A. nuda	oat	Cenrtal Asia	0-5000BC	cereal	ib/sd	allo 6x
Eleusine	Finger millet	East Africa	0-5000BC	cereal	ib/sd	4x
Hordeum	barley	E. Med.	before 5000BC	cereal	ib/sd	2x
Oryza sativa	Rice	Indo-China	0-5000BC	cereal	ib/sd	2x
O. glaberrina	Rice	West Africa	0-5000BC	cereal	ib/sd	2x
Pennisetum	bullrush millet	West Africa	0-5000BC	cereal	ob/sd	2x
Saccharum	Sugarcane	N. Guinea	0-5000BC	industrial	ob/cl	many x
Secale	Rye	Near East	0-5000BC	cereal	ob/sd	2x
Secalotriticum	Triticale	Mexico, Canada	after 1700BC	cereal	ib/sd	allo 6x
Sorghum	Sorghum	Trop. Africa	0-5000BC	cereal	ibob/sd	2x
Triticum monococcum	Eikhorn	Near East	before 5000BC	cereal	ib/sd	2x
T. timopheevii	Wheat	Near East	before 5000BC	cereal	ib/sd	allo 4x
T. turgidum	Emmer	Near East	before 5000BC	cereal	ib/sd	allo 4x
T. aestivum	bread wheat	Near East	before 5000BC	cereal	ib/sd	allo 6x
Zea	maize	C. America	0-5000BC	cereal	ob/sd	2x

ib - inbred/usually/always selfed, tolerant of inbreeding;
ob - outbred, habitually crossed, suffers inbreeding depression;
ibob - in-out-bred, usually nearer inbred than outbred;
cl - clonal; sd - seed propagated.

In South Africa, maize and wheat are the principle sources of carbohydrate and protein in the human diet. They also contribute to livestock production in the form of fodder. Their yield and quality have been greatly improved during the past several decades by traditional breeding methods. Further improvement will require more sophisticated breeding tools such as the employment of molecular diagnostic techniques to identify genotypes more accurately and speedily.

1.5 MOLECULAR MARKERS IN MAIZE

Maize (*Zea maize* 2n=2x=20) is an outcrossing species, domesticated from a teosinte (*Zea maize* spp *parviglumis*). Teosinte is a wild grass occurring naturally in isolated patches in Western Mexico (Allard, 1960; Poehlman, 1987; White and Doebley, 1998). The widespread use of hybrids in recent times, coupled with improved cultural practices by farmers, has more than tripled maize grain yields.

Electrophoretic separation of allelic variants of enzymes (allozymes) has in the past provided sufficient molecular markers for inter-varietal discrimination (Smith and Smith, 1991). Inbreeding and controlled selection has reduced the gene pool of maize considerably. This resulted in the requirement of more discriminating means of genotype assessment, such as the newer direct DNA assessment techniques.

Since the establishment of a linkage map in maize using RFLPs (Helentjaris, 1985), there has been a boom in molecular mapping and molecular applications in maize. RFLPs have been successfully used to discern between maize hybrids that could not be discriminated between with allozymes (Smith and Smith, 1991) and have become a preferred method to discriminate between highly homologous genotypes. RFLP probes of maize have also been used to study chromosomal evolution in maize and compared with other grass species (Ahn and Tanksley, 1993).

In comparative mapping studies, many authors have provided evidence of conserved linkage groups and duplicated loci in maize and other crops. Hulbert *et al.* (1990)

concluded that processes such as locus duplication were involved in the divergence of maize and sorghum. In comparative mapping of maize, wheat and rice, Ahn and Tanksley (1989) also concluded that the presence of orthologous loci, loci with similar phenotypes in different genera. These conserved linkage groups in grass species have played a major role in genome research by accelerating the predictions of DNA probe positions (Whitkus *et al.*, 1992; Ahn *et al.*, 1993).

RFLPs have been successfully used to establish genetic linkage to specific resistance genes in maize. To alleviate inconsistencies encountered in phenotypic scoring of resistance to *southern corn leaf blight* on mature plants in the field, a RFLP marker linked to *rhm*, a recessive gene for the disease, was established by Zaitlin *et al.* (1993). This marker is used widely today in breeding for disease resistance. In the same way, Sari-Gorla *et al.* (1996) was able to detect qualitative trait loci (QTLs) controlling *herbicidal tolerance* in maize for Alachlor with RFLPs. These loci did however not reveal significant association with tolerance in segregating populations.

PCR-based methods have also been used in linkage mapping and marker development. Oard and Dronavalli (1992) established that the RAPD technique could be used to screen individuals from genetic populations for linkage mapping.

With the advent of the AFLP technology, new applications have been found that were not possible with other fingerprinting techniques. Since AFLP is a high volume marker generator and covers a greater percentage of a genome, more informative markers have been generated. New applications such as the determination of testcross means (Melchinger *et al.*, 1998) and variances among F_3 progenies (Oard and Dronavalli, 1992) of F_1 crosses from test cross means and genetic distances of parents can now be done with greater accuracy using AFLP marker data. It is also possible to predict the relationship between hybrid performance and genetic diversity in maize with a higher degree of accuracy (Chin *et al.*, 1996).

1.6 MOLECULAR MARKERS IN WHEAT

Bread wheat (*Triticum aestivum* L. 2n=6x=42 [AABBDD]) is an allo-hexaploid with three related (homoeologous) genomes (A, B and D), each derived from a different ancestral genome (Poehlman, 1987). *Triticum monococcum* (2n=2x=14 [AA]) has been assumed to be the ancestor of the A genome of polyploid wheat. It was however found that the source of the A and B genomes was probably durum wheat (*Triticum turgidum* L. 2n=4x=28 [AABB]). Durum wheat was probably derived from a cross between *T. monococcum* and *Aegilops speltoides* (2n=2x=14 [BB]), a wild grass species. An additional genome was added by another grass, *Aegilops squarrosa* (2n=2x=14 [DD]), providing the D genome. The resulting triploid (2n=3x=21 [ABD]), produced a hexaploid though chromosome doubling.

The efficient use of molecular markers to assess diversity among elite, adapted germplasms of wheat has been hindered by the homoeology of hexaploid wheat (Brown-Guedira *et al.*, 1996). The large size of the genome (genomes A, B and D together) also contributes to the low level of inter-varietal polymorphism. Nullisomic-tetrasomic and disomic *T. aestivum* have been used to alleviate the problem of homoeology of hexaploid wheat. Van Deynze (1995) indicated easier detection of markers on homoeologous chromosomes using comparative maps of wheat chromosomes A, B and D with null-phenotypes. Ben Amer *et al.* (1997) mapped QTLs controlling different tissue-culture responses to chromosome 2B using a single chromosome substitution line for chromosome 2B. Another strategy to overcome the low level of polymorphisms in *T. aestivum* has been to use diploid wheat, *T. monococcum,* which provides higher levels of polymorphism and compliments and enriches *T. aestivum* maps (Dubcovsky *et al.*, 1996). However this is only with regards to the A genome of polyploid wheat.

The fact that the wheat ‘genome’ strictly consists of three genomes contributed by species that were closely related, requires a profiling technology that will show a high level of variation. Röder *et al.* (1995) showed that SSRs are abundant and variable and suitable molecular markers to use. Some researchers have however combined a number of fingerprinting technologies; Parker *et al.* (1998) made use of AFLP, RFLP and SSR fingerprinting to construct a partial genetic map for *T. aestivum*.

Another PCR profiling method that has been used with success is RAPD fingerprinting. Chen *et al.* (1998) identified markers linked to pathogenic resistance to *Puccina striiformis* f. sp. *tritici*. AFLP fingerprinting has also been used successfully to distinguish between methylation patterns in different organs in wheat (Donini *et al.*, 1997). Assessing the genetic diversity among wheat cultivars with AFLP fingerprinting has also proved to be more accurate (Barrett, 1998a; 1998b).

1.7 AIM

The aim of this investigation was to identify fragments in AFLP fingerprints that were associated with one maize and two wheat economically important traits, for their ultimate use in marker assisted selection.

The traits selected were:

- In maize, *gamete fertility/sterility,*
- In wheat *eye spot resistance/susceptibility* and
- In wheat *cytoplasmic male fertility/sterility.*

The project entailed the following components:

- Preparation of AFLP fingerprints.
- Identification of trait linked fragments - potential markers.
- Cloning and sequencing of potential markers – conversion of fragments into SCARs.
- Design of primers to amplify potential markers (SCARs).
- Validation of the suitability to use designed primers to amplify SCARs as markers linked to traits.

CHAPTER 2

WHEAT AND MAIZE AFLP FINGERPRINTS

2.1 INTRODUCTION

In this study, AFLP fingerprinting technology was used to identify putative polymorphic DNA markers linked to particular maize and wheat traits. In maize, near isogenic lines (NILs) conferring either *gamete sterility* or *fertility*, and the donor parent of the *gamete sterility* were compared. In wheat, NILs conferring *eye spot resistance* or *absence of resistance to eye spot* was compared to the donor parent carrying *eye spot resistance* genes. The second wheat trait, *cytoplasmic male sterility* was also investigated, of which the *male sterile wheat* line, a *male sterility restorer* line and a hybrid between these two lines were used.

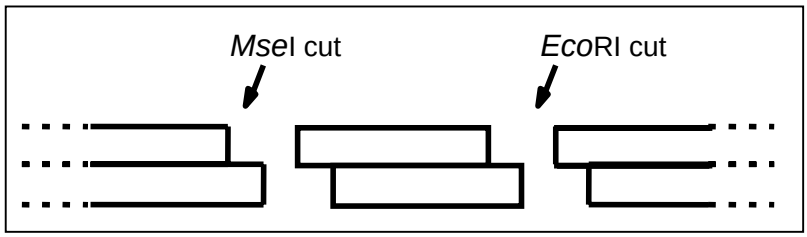
2.1.1 Summary of AFLP technology

AFLP is a DNA fingerprinting technique which detects DNA restriction fragments by means of PCR amplification (Vos *et al.*,1995; Lin and Kuo, 1997). The AFLP technology usually comprises of the following steps:

Restriction of the genomic DNA

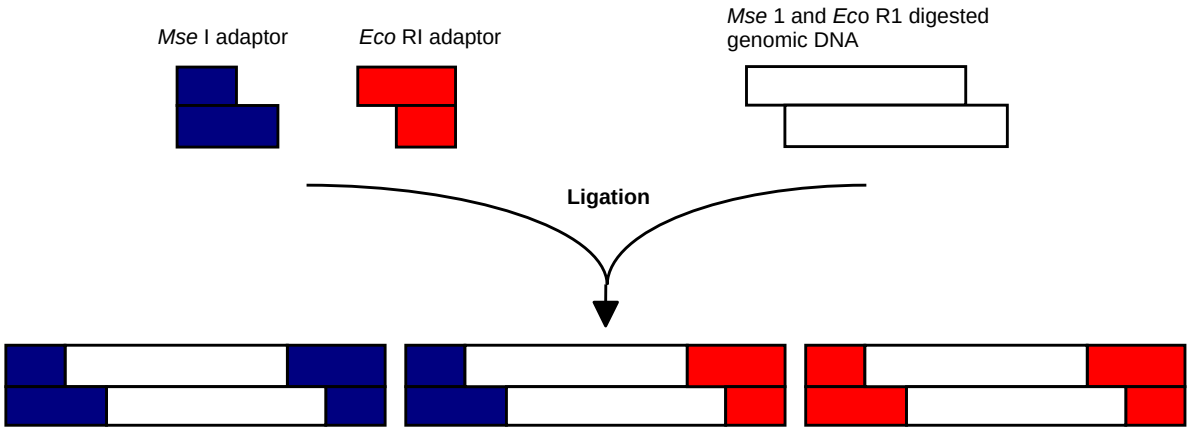
Restriction fragments of the genomic DNA are produced by using two different restriction enzymes: a frequent cutter (the four-base restriction enzyme *Mse*I) and a rare cutter (the six-base restriction enzyme *Eco*RI). Three types of restriction fragments are generated: fragments with *Eco*RI cuts at both ends,

fragments with *Eco*RI cut at one end and *Mse*I cut at the other end, and fragments with *Mse*I cuts at both ends.



Ligation of oligonucleotide adapters

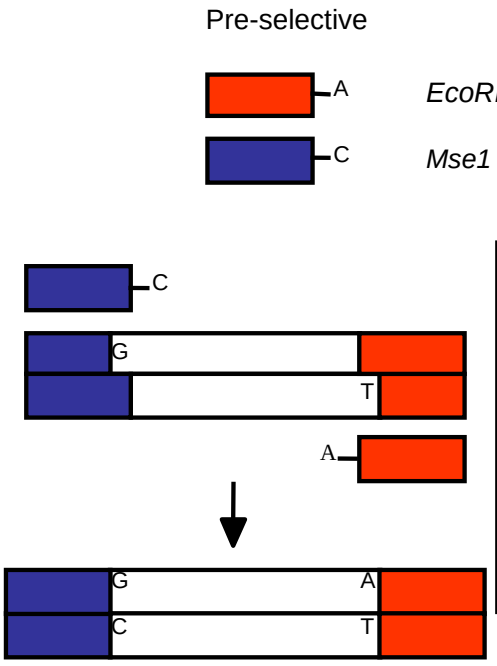
Double-stranded adapters consist of a core sequence and an enzyme-specific sequence. They are specific for either the *Eco*RI site or the *Mse*I site. Restriction and ligation take place in a single reaction. Ligation of the adapter to the restricted DNA alters the restriction site so as to prevent a second restriction from taking place after ligation has occurred.



Pre-selective amplification

Primers used in this step consist of a core sequence, an enzyme specific sequence and a selective single-base extension at the 3'-end. The sequences of the adapters and restriction sites serve as primer binding sites for the *pre-selective PCR amplification.* Each pre-selective primer has a *selective* nucleotide that will recognize the subset of restriction fragments having the matching nucleotide downstream from the restriction site.

The primary products of the pre-selective PCR are those fragments with one *Mse*I cut and one *Eco*RI cut, and also having the matching *selective* nucleotide. The pre-selective amplification step achieves a 16-fold reduction of the complexity of the fragment mixture.



EcoRI adapter+ selective base A

Mse1 adapter+ selective base C

a. Dilute restriction-ligation products.
b. Add pre-selective primers, dNTPs and Taq pol.
c. Perform PCR amplification.

Only those fragments with primer sites complementary to the additional bases will amplify.

This results in a 16-fold decrease in the complexity of the restriction-ligation products.

Selective amplification with labelled primers

Selective primers are either radio-labelled or fluorescently-labelled. They consist of an identical sequence to the pre-selection primers plus two

additional selective nucleotides at the 3'-end (i.e., a total of three selective nucleotides). These two additional nucleotides can be any of the 16 possible combinations of the four nucleotides. From the large number of fragments generated by the two restriction enzymes, only that subset of fragments having matching nucleotides at all three positions will be amplified at this stage (50-200 fragments). This step reduces the complexity of the PCR product mixture by 256 fold. Different primer combinations will generate different sets of fragments. Preliminary screening is used to choose primer pairs that generate suitable levels of variation for the taxa being studied. Additional selective nucleotides may be added for complex genomes, usually not more than a total of four selective nucleotides.

Visualisation and analysis of DNA fingerprints

In this investigation both autoradiography and silver staining were used to visualise the AFLP fingerprints.

2.2 MATERIALS AND METHODS

All buffers and solution compositions are detailed in Appendix A.

2.2.1 Plant material and DNA extraction

DNA was extracted from germinated maize and wheat seedlings. Maize seeds were germinated on wetted germination paper, rolled up, placed in plastic bags to reduce evaporation and left in the dark at 25°C for four days. Wheat seeds were germinated in the greenhouse for two weeks in pots containing a potting mix of soil and compost (1:1).

All the genotypes used in this investigation were supplied by courtesy of Sensako seed Production Company, detailed in table 2.1.

Table 2.1 Maize and wheat characteristics.

Plant	Trait	Code	Genotype	Trait
Maize	*Gamete sterility*	GASC	Donor parent of gamete sterility.	+
		GAS12	Displays gamete sterility	+
		GAS9	Gamete sterility absent	-
		GAS1	Gamete sterility absent	-
Wheat	*Eye spot resistance*	E9	Displays fungal resistance	+
		E10	Fungal resistance absent	-
		E12	Fungal resistance absent	-
		E13	Donor parent of fungal resistance	+
Wheat	*Cytoplasmic male sterility (CMS)*	C4	Cytoplasmic male sterile line	+
		C5	Cytoplasmic male sterile line	+
		C6	Restorer of CMS	-
		CR41	Restorer of CMS	-

Present (+)

Absent (-)

Standard steps of DNA extraction were followed for both maize and wheat with a few minor adjustments to extraction buffers (Table 2.2). DNA was extracted by destroying the cell walls, cell and nuclear membranes and then removing the polysaccharides and glycoproteins produced during the disruption process. For maize a less complex extraction buffer was used to extract maize DNA because the young maize coleoptiles did not contain secondary compounds such as phenolics or polysaccarides that are usually associated with older leaf material, as

was the case with the wheat DNA extraction.

Table 2.2 Steps and chemicals used to extract maize and wheat DNA.

Extraction steps	Maize	Wheat
Material	Coleoptiles	Two week old leaves
Cell wall disruption	Eppendorf tube grinder	Liquid nitrogen, mortar and pestle
Nuclear membrane disruption	SDS	CTAB
Chelating agent	EDTA	EDTA
Additional protective agents	2-Mercaptoethanol	PVP and 2-Mercaptoethanol
Protein and polysaccaride precipitation	SDS-protein-polysaccharide complex	Emulsification of proteins with phenol:chloroform, differential solution of polysaccharides in chloroform in the presence of CTAB
DNA precipitation	Isopropanol	Isopropanol
DNA wash	Removal of excess SDS and potassium acetate with 70% ethanol	Removal of excess PVP with 70% ethanol
DNA solution	1 X TE Buffer	1 X TE Buffer

DNA was extracted from maize coleoptiles by employing the procedure of Dellaporta, *et al*. (1983) with a few modifications. Doyle and Doyle's (1991) procedure, with a few modifications, was used to extract DNA from wheat leaves.

Maize DNA extraction

DNA was extracted from three one cm long coleoptiles. The three coleoptiles were cut into smaller pieces so that the pieces could fit into an eppendorf tube. The cell walls were disrupted by grinding the material with an eppendorf tube grinder. To the grindate, 400 µl maize extraction buffer and 30 µl 20 % SDS were added. The solution was mixed and incubated at 65°C for 10 minutes. 135 µl potassium acetate (pH5,2) was added to the incubated mixture, mixed and placed on ice for 10 minutes. This mixture was centrifuged for 10 minutes in a bench centrifuge and the supernatant transferred to an eppendorf tube. 0.6 volumes (600 µl) of isopropanol were added to the supernatant, and the tube inverted a few times to ensure proper mixing. As soon as DNA became visible, it was spooled out with a glass hook. In cases, where the DNA was not visible, the tube was centrifuged for 10 minutes in a bench centrifuge. The DNA pellet was washed in 500 µl ice-cold 70 % ethanol and precipitated by centrifugation in a bench centrifuge for five minutes. The pellet was suspended in 200 µl 1 x TE buffer and left overnight at 4°C. The concentration of the DNA was determined by loading three µl of the DNA solution on an agarose gel containing one µg/ml of ethidium bromide.

Wheat DNA extraction

DNA was extracted from two-week-old wheat leaves. Four leaves were frozen at a time in 10 ml liquid nitrogen in a mortar, while grinding with a pestle to disrupt the cell walls. Three millilitres of preheated CTAB (60°C) were added to the grindate and homogenised by further grinding. The residual grindate in the mortar was rinsed into the corex tube with 1 ml CTAB buffer. This mixture was incubated at 60°C for one hour with occasional gentle swirling. The corex tube was cooled at room temperature for 15 minutes. The DNA was extracted by adding an equal volume (four ml) of chloroform : isoamyl alcohol (24 : 1) and left for five minutes at room temperature, thereafter centrifuged for 10 minutes at 7719 g. The top, DNA containing layer was removed by pipetting into a corex tube. One volume (four ml)

of cold isopropanol was added and mixed gently to precipitate the nucleic acids and the visible DNA spooled out of solution with a glass hook. In instances when the DNA did not become visible, the solution was centrifuged at 12 062 g to precipitate the DNA. The DNA was finally washed in 500 µl of 70 % ethanol and precipitated by centrifugation at 12 062 g. The ethanol was poured off and the pellet dried in a speedy vacuum, after which the pellet was resuspended in 300 µl 1 x TE buffer and dissolved overnight at 4°C.

2.2.2 DNA purification

The GENECLEAN II kit (Bio 101, Inc., Cat # 1001-400) was used to purify maize and wheat DNA by following the manufacturer's reaction conditions. Three volumes (300 µl) sodium iodide (NaI) and five µl glass milk suspension were added to 100 µl of the extracted genomic DNA suspension (±1 µg DNA) and incubated for five minutes on ice with occasional inversion of the tube to keep the glass milk in suspension. The sodium iodide causes selective binding of the DNA to the silicone matrix. The glass milk-DNA complex was collected in an eppendorf tube by centrifugation for five minutes. The pellet was washed three times with 500 µl *New Wash* solution by centrifugation to eliminate RNA and proteins. After the third wash, the pellet was resuspend in 10 µl of 1 X TE buffer and incubated at 55°C for 15 minutes to separate the DNA from the silicone matrix. Finally the solution was centrifuged for one minute and the supernatant collected in an eppendorf tube. The DNA concentration was determined by comparing three µl of the DNA extract and 100 ng of lambda DNA on a 1 % agarose gel.

2.2.3 Restriction of genomic DNA

The AFLP core reagent kit (GibcoBRL; Cat. No. 10482-016) was used for the digestion step. Included in the kit was tomato DNA, which was used as the control to which the maize and wheat digestions could be compared to establish whether the digestions were complete. A 250 ng of purified DNA (either maize or wheat),

five µl of 5 x reaction buffer and two µl *EcoRI* / *MseI* enzyme reaction mixture was used, which was made up to a final volume of 25 µl with ddH_2O in an eppendorf tube. The mixture was collected by brief centrifugation in a bench centrifuge and thereafter incubated for two hours at 37°C. The mixture was finally incubated for 15 minutes at 70°C to inactivate the restriction enzymes and immediately placed on ice. The reaction products were collected by brief centrifugation in a bench centrifuge.

2.2.4 Ligation of adapters to restricted genomic DNA

Twenty four microliters adapter ligation solution and one µl T4 DNA ligase were added to the *EcoRI* and *MseI* digested DNA (prepared according to the specifications in section 2.2.3). The reaction was collected by brief centrifugation and incubated at 20°C for two hours. A 1 : 10 dilution of the ligation reaction was prepared in a 100 µl volume by adding 10 µl of the ligation mix to 90 µl of ddH_2O.

2.2.5 Pre-amplification

In the pre-amplification protocol, 40 µl pre-amplification primer mix, five µl 10 X PCR buffer for AFLP and one µl *Taq* DNA polymerase were added to five µl diluted ligation mix of maize or wheat in an eppendorf. The solution was gently mixed and the reaction collected by brief centrifugation and then overlaid with two drops of mineral oil. Twenty amplification cycles were performed in a Hybaid Omnigene TR3 CM220 thermocycler consisting of 30 s at 94°C, 60 s at 56°C and 60 s at 72°C. After the PCR cycling reactions, the samples were removed from the thermocycler and diluted 1 : 50.

2.2.6 Primer labelling

For the radioactive detection of the AFLP profiles, the 5’ end of the *Eco*RI primers

had to be labelled with [γ-^{32}P]ATP. Primer labelling was performed by phosphorylating the 5' end of the *Eco*RI primers with [γ-^{32}P]ATP and T4 polynucleotide kinase. For ^{32}P-labelling, 10.8 µl *Eco*RI primer, three µl 5 X kinase buffer, 12 µl [γ-^{32}P] ATP (3 000 Ci/mmol) and 1.2 µl T4 polynucleotide kinase (10 units/µl) were added to an eppendorf tube and made up to a final volume of 30 µl with ddH_2O and then mixed gently. The tube was centrifuged briefly to collect the contents and incubated at 37°C for one hour. The tube was then briefly centrifuged and placed on ice. No additional cleaning steps were performed.

This step was excluded in the case where silver staining of PAGE gels were prepared.

2.2.7 Selective amplification

Depending on which visualisation procedure will be used, primers of the selective amplification may require labelling. In this investigation the maize *gamete sterility* DNA fingerprints were visualised with radioactivity, but the wheat DNA fingerprints were visualised with both radioactivity and silver (which does not require primer labelling). Profiles used for the determination of percentage polymorphic DNA in maize *gamete sterility* and wheat *eye spot resistance* were radio labelled, and for identification of fragments potentially linked to wheat *eye spot resistance*, silver staining was used. For wheat *cytoplasmic male sterility*, only silver staining was used.

The primers used were selected according to criteria provided by the AFLP starter primer kit (GibcoBRL; Cat. No. 10483-014) and are listed in table 2.3.

Table 2.3 Primer pairs utilised for AFLP fingerprints.

Trait	Primer pairs					
	GC content (%)					
	44,7	**46,3**	**47,4**	**48,7**	**50,0**	**51,3**
Maize *gamete sterility* **primer pairs**	M-CTA / E-AAC M-CTT / E-ACC M-CAT / E-ACA M-CTA / E-ACA M-CTT / E-ACA M-CAA / E-ACT M-CAT / E-ACT M-CTA / E-ACT		M-CAG / E-AAC M-CTC / E-AAC M-CTG / E-AAC M-CAC / E-ACA M-CTG / E-ACA M-CAG / E-ACT M-CTC / E-ACT M-CAT / E- AGG M-CTA / E-AGG M-CAC / E-AAC		M-CAC / E-AGG M-CTG / E-AGG M-CTG / E-AGC M-CAG / E-AGC	
Wheat *eye spot resistance* **primer pairs**	M-CAA / E-AAC M-CAT / E-AAC M-CTA / E-AAC M-CTT / E-AAC M-CAA / E-ACA M-CAT / E-ACA M-CTA / E-ACA M-CTT / E-ACA M-CAA / E-ACT		M-CAC /E-AAC M-CAG / E-AAC M-CTC / E-AAC M-CTG / E-AAC M-CAC / E-ACA M-CAG / E-ACA M-CTC / E-ACA M-CTG / E-ACA M-CAC / EACT		M-CAC / E-AGG M-CAG / E-AGG M-CTC / E-AGG M-CTG / E-AGG M-CAC / E-AGC M-CAG / E-AGC M-CTC / E-AGC M-CTG / E-AGC M-CAC / E-ACC	

	M-CAT / E-ACT		M-CAG / E-ACT		M-CAG / E-ACC	
	M-CTA / E-ACT		M-CTC / E-ACT		M-CTC / E-ACC	
	M-CTT / E-ACT		M-CTG / E-ACT		M-CTG / E-ACC	
			M-CAC / E-AGG			
			M-CAT / E-AGG			
			M-CTA / E-AGG			
			M-CTT / E-AGG			
			M-CAA / E-AGC			
			M-CAT / E-AGC			
			M-CTA / E-AGC			
			M-CTT / E-AGC			
			M-CAA / E-ACC			
			M-CAT / E-ACC			
			M-CTA / E-ACC			
			M-CTT / E-ACC			
			M-CTT / E-ACG			
Wheat *cytoplasmic male sterility* primer pairs	M-CAA / E-ACA	M-CAT / E-AGCA	M-CAG / E-AAC	M-CAT / E-AGCG	M-CAG / E-AGG	M-CAC / E-AGCG
	M-CAT / E-ACA	M-CAT / E-ACAC	M-CAC / E-ACA	M-CAC / E-AGCA	M-CTG / E-AGG	M-CAG / E-AGCG
	M-CAA / E-ACT	M-CAT / E-ACTC	M-CAG / E-ACA	M-CAG / E-AGCA	M-CAG / E-AGC	
			M-CTC / E-ACA	M-CAC / E-ACAC	M-CTC / E-AGC	
			M-CAG / E-ACT	M-CAG / E-ACAC	M-CAG / E-ACC	
			M-CTG / E-ACT	M-CAC / E-ACTC		
			M-CTT / E-AGG	M-CAG / E-ACTC		
			M-CAA / E-AGC			
			M-CTA / E-AGC			
			M-CAT / E-AGC			

M = *Mse*I primer sequence, namely, 5'-GAT GAG TCC TGA GTA A-3'

E = *Eco*RI primer sequence, namely, 5'-GAC TGC GTA CCA ATT C-3'

Reaction conditions

Standard reaction conditions as recommended by the supplier were used in the case of maize *gamete sterility.* For each primer pair in the standard reaction, 0,5 µl *Eco*RI primer and 4,5 µl *Mse*I primer, as well as two µl 10 X PCR buffer for AFLP and one µl *Taq* DNA polymerase made up to 20 µl with ddH_2O, were placed in an 0,2 ml PCR tube. Five µl diluted template DNA (1 : 50 dilutions of the pre-amplification step) were added, the reaction mixed gently and centrifuged briefly to collect the reaction.

However, in the case of wheat standard reaction conditions were not available at the time of this work and a number of different reaction combinations were tested (Table 2.4).

Table 2.4 Optimisation conditions tested for selective amplification of wheat DNA.

Reaction components	**Reaction mixtures in µl**					
	1[1]	**2**	**3**	**4**	**5**	**6**
DNA	5	5	5	5	5	5
*Eco*R1 primer (27,8 ng/µl)	0,5	0,5	0,5	0,25 *	0,5	0,5
*Mse*1 primer (6,7 ng/µl including dNTP's)	4,5	4,5	4,5	4,5	8*	4,5
10 x PCR buffer for AFLP	2	2	2	2	2	2
Taq DNA polymerase	0,1	0,1	0,2 *	0,1	0,1	0,2 *
dNTP's (200 mM each)	0	2 *	0	0	0	2 *
ddH_2O	6,9	4,9	6,8	7,15	2,9	4,8
Total	20					

[1] Standard maize reaction.

* Indicates which component of the standard maize reaction was altered.

The best results for wheat *cytoplasmic male sterility* were obtained with the same reaction conditions used for maize and therefore applied in all subsequent wheat *cytoplasmic male sterility* reactions (Figure 2.1). It should however be noted that in the case of maize *gamete sterility* the primers were labelled, but unlabelled for wheat *cytoplasmic male sterility.*

In the case of wheat *eye spot resistance* the standard maize reaction conditions did not yield favourable results. It was found that reaction mixture 6 (Table 2.4) produced the most favourable results and was applied in all subsequent reactions (Figure 2.2). The reaction conditions involved 0,5 µl *Eco*RI primer (radio labelled as well as unlabelled) and 4,5 µl *Mse*I primer, as well as 2 µl 10 X PCR buffer for AFLP, 2 µl 200 mM of each dNTP's and 2 µl *Taq* DNA polymerase made up to 20 µl with ddH_2O, that were placed in an 0,2 ml PCR tube. Five µl diluted template DNA (1 : 50 dilutions of the pre-amplification step) was added, the reaction mixed gently and centrifuged briefly to collect the reaction.

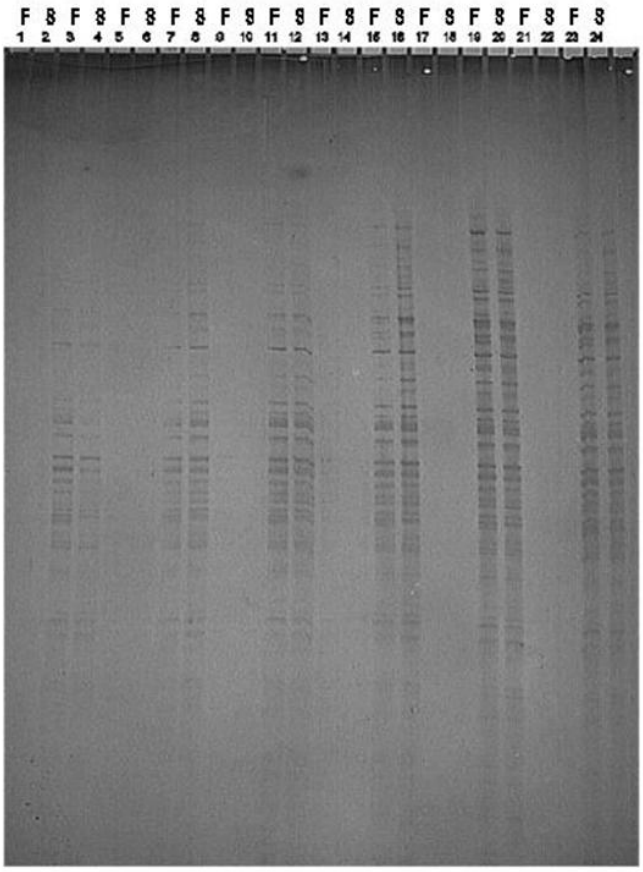


Figure 2.1 Optimisation of selective amplification for wheat *cytoplasmic male sterility*. F indicates the *cytoplasmic male fertile* genotype C4 and S indicates the *cytoplasmic male sterile* genotype C6.

Lanes 3 and 4:	Standard thermocycler conditions of *maize gamete sterility*.
Lanes 7 and 8:	Increased annealing time during touchdown phase.
Lanes 11 and 12:	Increased annealing time during amplification phase.
Lanes 15 and 16:	Combined increase in annealing time in both touchdown and amplification phase.
Lanes 19 and 20:	Combined increase in annealing and extension time in amplification phase.
Lanes 23 and24:	Extended extension time.
Lanes 1; 2; 5; 6; 9; 10; 13; 14; 17; 18; 21 and 22:	Open.

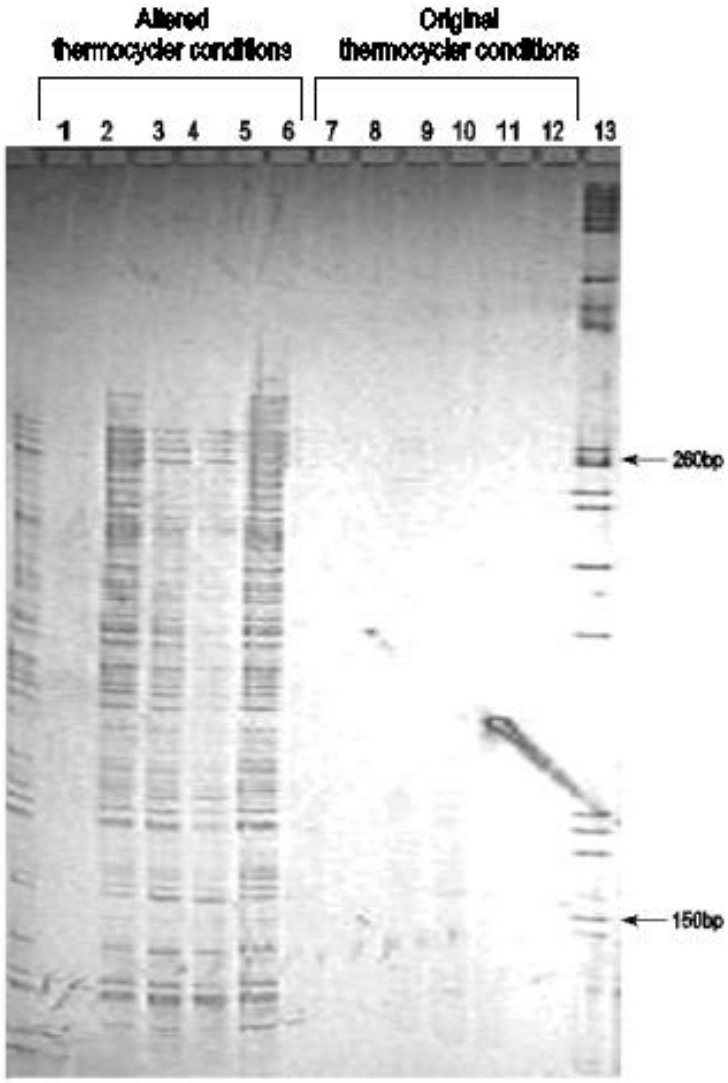


Figure 2.2 Optimisation of selective amplification for wheat *eye spot resistance.*

Lane 1 and 7:	Open.
Lane 2 and 8:	Standard reaction plus 200 mM dNTP's.
Lane 3 and 9:	Original reaction plus an extra unit of enzyme.
Lane 4 and 10:	Original reaction plus stabilizer and half the amount *Eco*RI primer.
Lane 5 and 11:	Original reaction plus twice the amount of *Mse*I primer.
Lane 6 and 12:	Original reaction plus two units enzyme plus 200 mM dNTP's.
Lane 13:	Molecular weight marker – Lambda DNA digested with *Pst*I.

Amplification

The reaction mixture was overlaid with 2 drops of mineral oil and the amplification carried out in a Hybaid Omnigene TR3 CM220 thermocycler. In the case of maize, the initial cycles consisted of 30 s at 94°C, 30 s at 65°C and 60 s at 72°C followed by a touch down phase of 10 cycles. The touch down phase started at cycle two with 30 s at 94°C, 30 s at 65°C and 60 s at 72°C, decreasing the annealing temperature with one degree per cycle until it reached cycle ten with 30 s at 94°C, 30 s at 56°C and 60 s at 72°C. The following 23 cycles consisted of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C.

In the case of wheat the optimal cycling conditions were initially established by testing a number of different cycling options (Table 2.5).

Table 2.5 Thermocycler profiles tested for the optimisation of selective amplification of wheat genotypes.

No. cycles	Profile					
	0[1]	**1**	**2**	**3**	**4**	**5**
Cycle 1	**30s @94°C** **30s @65°C** **60s @72°C**	60s @94°C 90s@65°C 90s @72°C	60s @94°C 60s @65°C 90s @72°C	60s @94°C 90s @65°C 90s @72°C	**30s @94°C** 60s @65°C 90s @72°C	60s @94°C 60s @65°C 90s @72°C
Cycle 2	**30s @94°C** **30s @64°C** **60s @72°C**	60s @94°C 90s@64°C 90s @72°C	60s @94°C 60s @64°C 90s @72°C	60s @94°C 90s @64°C 90s @72°C	**30s @94°C** 60s @64°C 90s @72°C	60s @94°C 60s @64°C 90s @72°C
Cycle 3	**30s @94°C** **30s @63°C** **60s @72°C**	60s @94°C 90s@63°C 90s @72°C	60s @94°C 60s @63°C 90s @72°C	60s @94°C 90s @63°C 90s @72°C	**30s @94°C** 60s @63°C 90s @72°C	60s @94°C 60s @63°C 90s @72°C
Cycle 4	**30s @94°C** **30s @62°C** **60s @72°C**	60s @94°C 90s@62°C 90s @72°C	60s @94°C 60s @62°C 90s @72°C	60s @94°C 90s @62°C 90s @72°C	**30s @94°C** 60s @62°C 90s @72°C	60s @94°C 60s @62°C 90s @72°C

Cycle 5	**30s @94°C** **30s @61°C** **60s @72°C**	60s @94°C 90s@61°C 90s @72°C	60s @94°C 60s @61°C 90s @72°C	60s @94°C 90s @61°C 90s @72°C	**30s @94°C** 60s @61°C 90s @72°C	60s @94°C 60s @61°C 90s @72°C
Cycle 6	**30s @94°C** **30s @60°C** **60s @72°C**	60s @94°C 90s@60°C 90s @72°C	60s @94°C 60s @60°C 90s @72°C	60s @94°C 90s @60°C 90s @72°C	**30s @94°C** 60s @60°C 90s @72°C	60s @94°C 60s @60°C 90s @72°C
Cycle 7	**30s @94°C** **30s @59°C** **60s @72°C**	60s @94°C 90s@59°C 90s @72°C	60s @94°C 60s @59°C 90s @72°C	60s @94°C 90s @59°C 90s @72°C	**30s @94°C** 60s @59°C 90s @72°C	60s @94°C 60s @59°C 90s @72°C
Cycle 8	**30s @94°C** **30s @58°C** **60s @72°C**	60s @94°C 90s@58°C 90s @72°C	60s @94°C 60s @58°C 90s @72°C	60s @94°C 90s @58°C 90s @72°C	**30s @94°C** 60s @58°C 90s @72°C	60s @94°C 60s @58°C 90s @72°C
Cycle 9	**30s @94°C** **30s @57°C** **60s @72°C**	60s @94°C 90s@57°C 90s @72°C	60s @94°C 60s @57°C 90s @72°C	60s @94°C 90s @57°C 90s @72°C	**30s @94°C** 60s @57°C 90s @72°C	60s @94°C 60s @57°C 90s @72°C
Cycle 10	**30s @94°C** **30s @56°C** **60s @72°C**	60s @94°C 90s@56°C 90s @72°C	60s @94°C 60s @56°C 90s @72°C	60s @94°C 90s @56°C 90s @72°C	**30s @94°C** 60s @56°C 90s @72°C	60s @94°C 60s @56°C 90s @72°C
Cycles 11-34	**30s @94°C** **30s@56°C** **60s @72°C**	**30s @94°C** **30s@56°C** **60s @72°C**	**30s @94°C** 60s@56°C **60s @72°C**	**30s @94°C** 60s@56°C **60s @72°C**	**30s @94°C** 60s@56°C 90s @72°C	**30s @94°C** **30s@56°C** **60s @72°C**
Cycles 35	-	-	-	-	-	5min@72°C

The bold lettering indicates the unaltered phases.

[1] Standard thermocycler profile used in maize *gamete sterility.*

It was found that the following produced the best results. In the case of wheat *eye spot resistance*, the PCR profile of the standard reaction used in maize gave the most favourable results. In the case of wheat *cytoplasmic sterility*, PCR profile 4 gave the most favourable results. The initial cycles consisted of 60 s at 94°C, 60 s

at 65°C and 90 s at 72°C followed by a touch down phase of 10 cycles. The touch down phase started at cycle two with 30 s at 94°C, 60 s at 65°C and 90 s at 72°C, decreasing the annealing temperature with one degree per cycle until it reached cycle ten with 30 s at 94°C, 30 s at 56°C and 60 s at 72°C. The following 23 cycles consisted of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C.

After PCR amplification, the samples were removed from the thermocycler and 20 µl of sequencing gel loading buffer was added. The samples were denatured at 90°C for three minutes and placed on ice immediately. The samples were then loaded on a 6 % polyacrylamide gel and run for 2½ hours at 70 Watt.

2.2.8 Separation of DNA fragments

DNA fragments were separated either by agarose or by polyacrylamide gel electrophoresis (PAGE). The advantage of using agarose gels are that a large range of fragment sizes can be separated and it is easier to handle than a polyacrylamide gel. Polyacrylamide can separate fragments that differ in one nucleotide in size, however the highest resolution obtained with agarose is four nucleotides. PAGE is therefore more effective to separate fragments of DNA with small differences. Both these separation strategies were employed and their use depended on the sizes of the fragments that needed to be separated.

In the case of PAGE, six percent polyacrylamide gels were prepared to discriminate between DNA fragments that differ in sizes by one base pair. It was poured between two glass plates, and after polymerisation the DNA fragments were separated by electrophoresis.

2.2.9 Silver staining of DNA

The photochemical derived reaction of silver staining was used to detect nanogram amounts of nucleic acids without radioactivity. In this reaction, silver bound to the nucleic acid bases, was then reduced selectively under alkaline conditions by formaldehyde to form metallic silver (Chalhoub *et al.*, 1997) and precipitated making it possible to visualise the DNA**.** Promega's silver staining system (Cat. No. DQ7050) was used to detect the DNA fragments in polyacrylamide gels.

The gel was firstly immersed in one litre fix/stop solution followed by gentle agitation. The fix/stop solution was then poured off and saved for later use. The gel was washed three times in one litre deionised water with gentle agitation. Pre-cooled staining solution (one litre) was added and mixed gently for 45 minutes. To dispose of excess stain, the gel was quickly rinsed with deionised water. Developer solution pre-cooled at 4°C (one litre) was added, and gently agitated until the fragments became visible (two - five minutes). The fix/stop solution was added directly to the developer followed by agitation for five minutes to stop the reaction. The gel was rinsed with one litre deionised water before it was analysed.

2.2.10 Preparation of autoradiographs

In the cases where radiolabelled primers were used in order to visualise the DNA through autoradiography, the PAGE gels were prepared in the regular way between glass plates separated by a wedge. The gel for autoradiography was removed from the plate by placing large piece of Whatmann paper over the gel and gently lifting the gel from the plate. The gel was dried in a vacuum drier for one hour at 80ºC. The dried gel was exposed to X-ray film for 48 hours. The X-ray film was developed according to the manufacturers instructions (Kodak). The film was developed for five minutes in developer, rinsed in dH_2O for five minutes, and fixed for three minutes in fixer. The film was then rinsed for five minutes under running H_2O and left to dry for analysis.

2.3 RESULTS

2.3.1 DNA yield at initial stages of the AFLP fingerprint procedure

After DNA extraction

The genomic DNA yields of maize varied from 5 µg to 15 µg, while for wheat it ranged between 15 µg and 39 µg.

After restriction digestion

The sizes of the digested fragments were determined by separating the fragments in 1,8 % agarose gels. The separated DNA fragments of both maize and wheat digested with *Eco*RI yielded DNA fragments that ranged in size from 23 130 bp to 1 357 bp in size. However, the size range of DNA fragments digested with both *Eco*RI and *Mse*I ranged from 5 148 bp to 100 bp (Figure 2.3). Smaller fragments may have been present, but due to the resolution power of agarose could not be discerned.

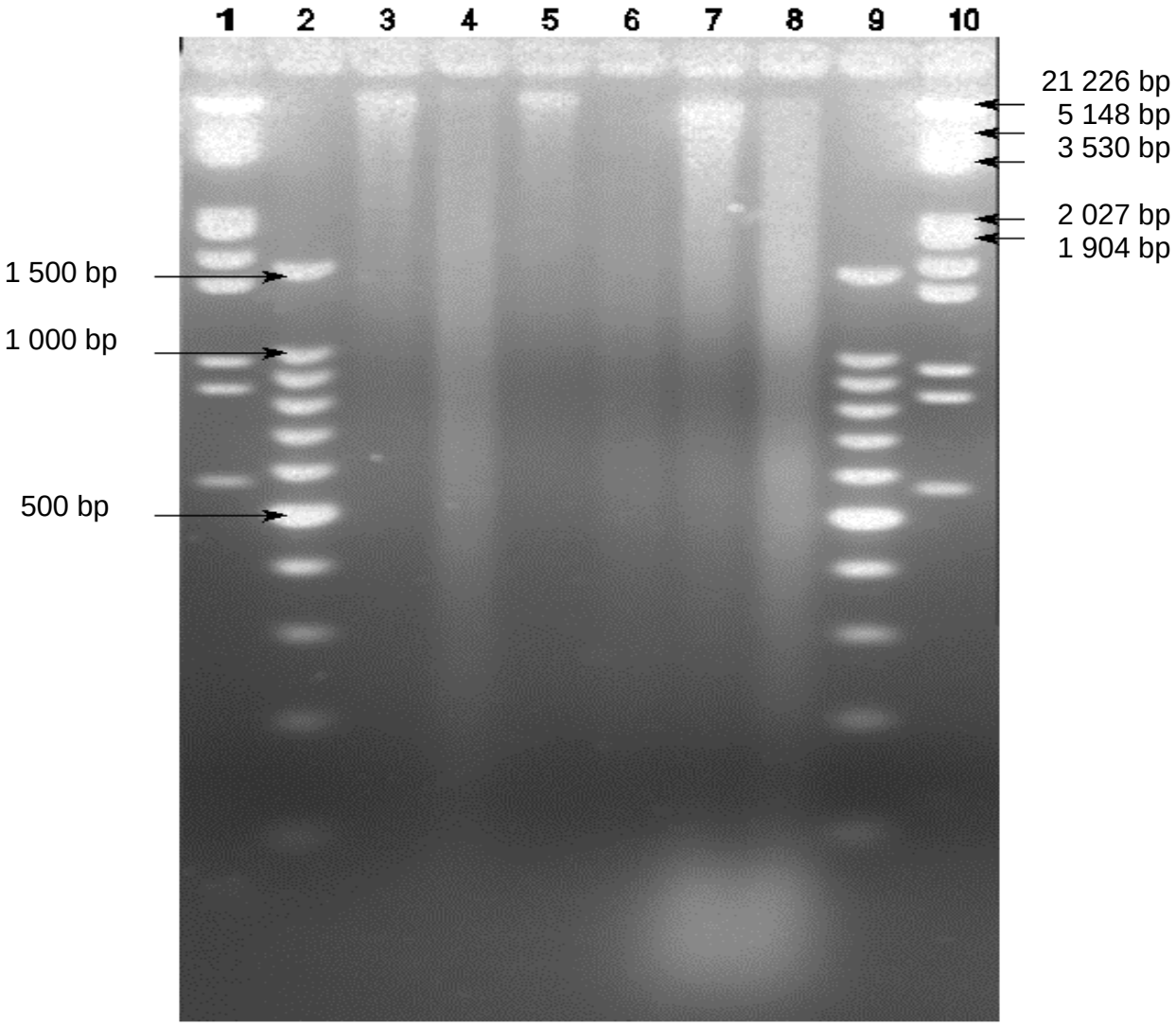


Figure 2.3 Fragment sizes of restriction digestion by *Eco*RI and *Mse*I

Lane 1	Molecular weight marker III (Boehringer Mannheim).
Lane 2	100 bp DNA ladder (Promega).
Lane 3	Tomato genomic DNA digested with *Eco*RI (control).
Lane 4	Tomato genomic DNA digested with *EcoRI* and *MseI* (control).
Lane 5	Maize genomic DNA digested with *Eco*RI.
Lane 6	Maize genomic DNA digested with *Eco*RI and *Mse*I.
Lane 7	Wheat genomic DNA digested with *Eco*RI.
Lane 8	Wheat genomic DNA digested with *Eco*RI and *Mse*I.
Lane 9	100 bp DNA ladder (Promega).

Lane 10 Molecular weight marker III (Boehringer Mannheim).

Pre-amplification products

The range of fragments of maize and wheat after pre-amplification were similar. The fragments ranged from 831 bp to less than 100 bp. However, a high concentration of fragments at about 600 bp was detected in wheat pre-amplification, but not in the maize pre-amplification (Figure 2.4).

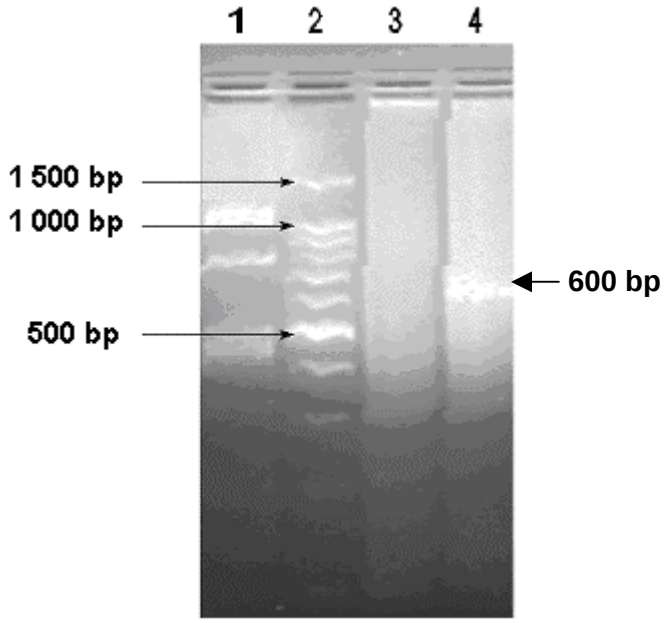


Figure 2.4 Fragment sizes of pre-amplified adapter ligated genomic DNA.

Lane 1 Molecular weight marker III (Boehringer Mannheim).
Lane 2 100 bp DNA ladder (Promega).
Lane 3 Pre-amplified adapter ligated genomic maize DNA.
Lane 4 Pre-amplified adapter ligated genomic wheat DNA.

2.3.2 AFLP fingerprints

Primer performance

Both silver staining and autoradiography were used to visualise the AFLP fingerprints in polyacrylamide gels (Table 2.6).

Table 2.6 AFLP fingerprinting visualisation procedures used.

Trait	**Maize** ***gamete sterility***	**Wheat** ***eye spot resistance.***	**Wheat** ***cytoplasmic male sterility***
Fingerprint visualisation	Radioactivity	Radioactivity Silver staining	Silver staining

Fragments of the AFLP fingerprints that ranged in size from 50 bp to 200 bp were used to determine the percentage polymorphic DNA (Figure 2.5) according to the following formula:

$$\textit{Percentage polymorphic DNA} = \frac{100 - \text{average number of monomorphic fragments}}{\text{average total number of fragments}}$$

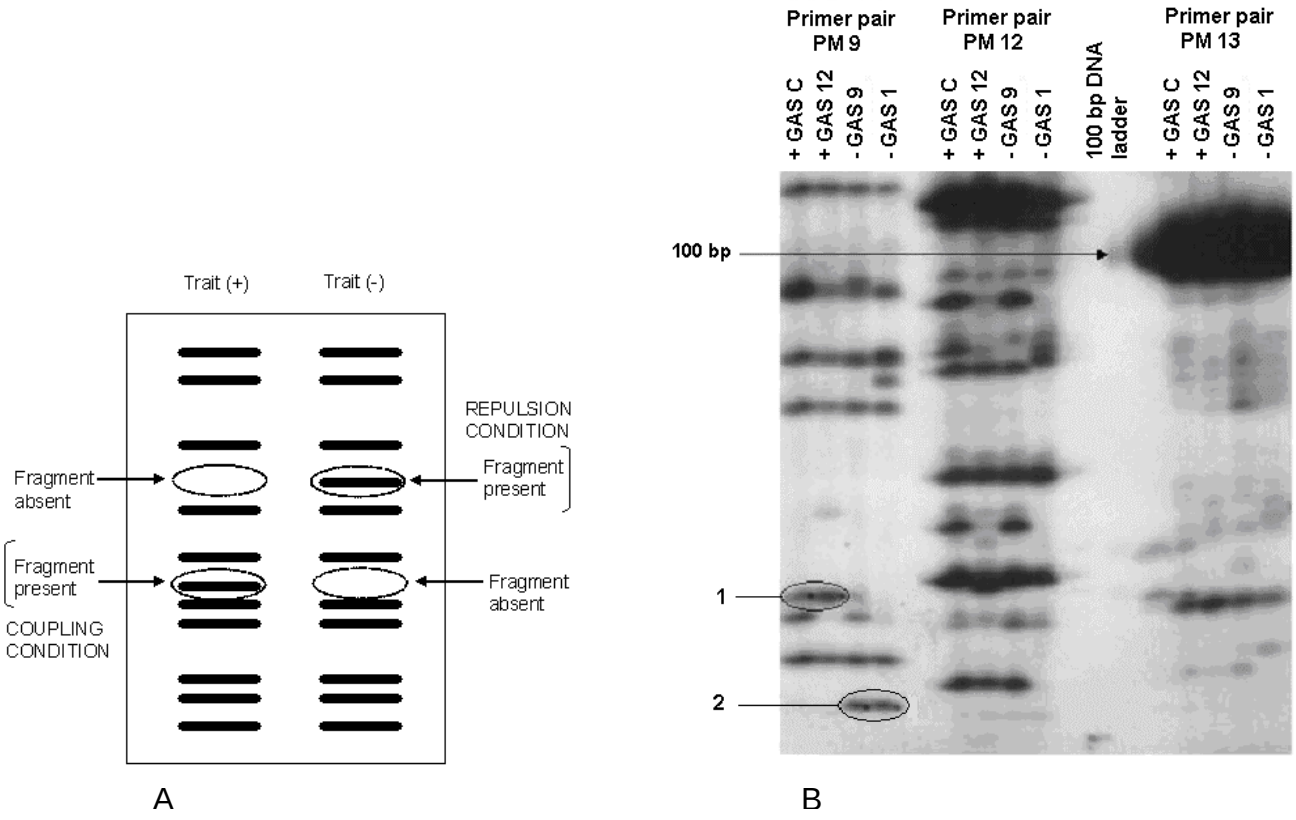


Figure 2.5 Polymorphic loci. A: Coupling (present in trait containing genotype) and repulsion coupling (not present in trait containing genotype) conditions. B: AFLP fingerprint of maize *gamete sterility.* Numbers 1 and 2 indicate the polymorphic fragments.

Sixteen of the 22 primer pairs for maize *gamete sterility*, 27 of the 48 wheat primer pairs for wheat *eye spot resistance* and 5 of 29 of the primer pairs of *cytoplasmic male sterility* were suitable for these calculations.

Overall, maize yielded less fragments than wheat. The fragment yield per primer combination for maize *gamete sterility* ranged from 45 to 69 fragments with an average yield of 60 fragments (Table 2.7), while for wheat *eye spot,* the average yield was 99 fragments, which ranged between 69 and 150 fragments per primer combination (Table 2.8). In the case of wheat *cytoplasmic male sterility* the average yield was 42 and ranged between 51 and 34 (Table 2.9).

Table 2.7 Useful AFLP primer products relative to the primer GC content of the primer pairs used in maize *gamete sterility.*

GC %	Number of useful primer combinations	Number of fragments			Number of monomorphs			Percentage polymorphic DNA
		Avg.	High	Low	Avg.	High	Low	
44,7	3	67,0	69	63	38,0	42	33	43,3
47,4	9	57,6	69	45	24,5	29	16	57,5
50	4	61,0	66	54	23,0	30	18	63,0

Avg. = average number of fragments identified per gel.

High = highest number of fragments identified per gel.

Low = lowest number of fragments identified per gel.

Table 2.8 Useful AFLP primer products relative to the primer GC content of the primer pairs used in wheat *eye spot resistance.*

GC %	Number of useful primer combinations	Number of fragments			Number of monomorphs			Percentage polymorphic DNA
		Avg.	High	Low	Avg.	High	Low	
44,7	6	95,5	115	85	71,6	89	62	25,0
47,4	11	101,0	150	69	69,7	100	43	31,0
50	11	99,9	120	74	70,5	110	50	29,4

Avg. = average number of fragments identified per gel.

High = highest number of fragments identified per gel.

Low = lowest number of fragments identified per gel.

Table 2.9 Useful AFLP primer products relative to the primer GC content of the primer pairs used in wheat *cytoplasmic male sterility.*

GC %	Number of useful primer combinations	Number of fragments			Number of monomorphs			Percentage polymorphic DNA
		Avg.	High	Low	Avg.	High	Low	
48,7	3	43	49	40	39	45	36	9,3
50,0	1	51	51	51	45	45	45	11,7
51,3	1	34	34	34	29	29	29	14,7

Avg. = average number of fragments identified per gel.

High = highest number of fragments identified per gel.

Low = lowest number of fragments identified per gel.

It should be noted that when the primer performances were compared in terms of their GC content distinct differences were identified between maize *gamete sterility* and wheat *eye spot resistance*. In maize *gamete sterility*, primers with a higher GC content yielded on average more polymorphic DNA (Table 2.7), while for wheat *eye spot resistance* the polymorphic DNA seems to increase to a certain point and then decrease again (Table 2.8). Although no useful AFLP profiles of wheat *cytoplasmic male sterility* with the same GC content as maize *gamete sterility* and wheat *eye spot resistance* could be obtained, the values for this trait also indicates an increase in polymorphic DNA with an increase in GC content (Table 2.9).

Comparative analysis of 3-nucleotide and 4-nucleotide selective primers

Generally it is known that when additional nucleotides are added to the 3' end of the selective primer, the resulting fingerprint changes as well. Wheat fingerprints generated with selective primers with three selective nucleotides were compared to those generated with selective primers with four selective nucleotides. It was observed that some of the fingerprints remained rather similar, while in some new fragments either appeared or disappeared at specific size positions (Figure 2.6).

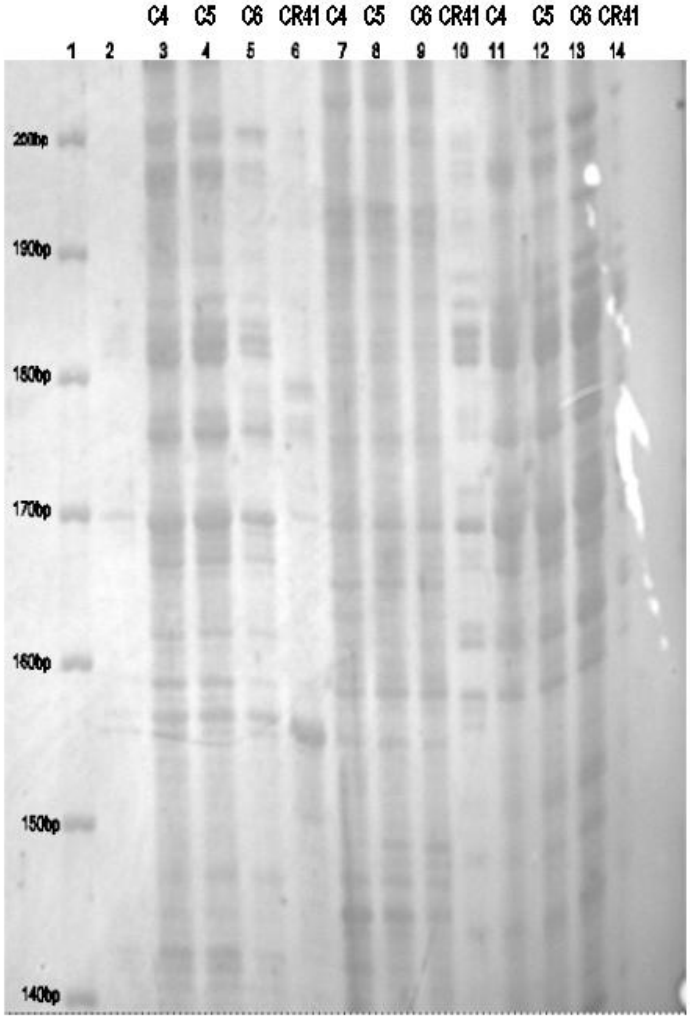


Figure 2.6 Comparison between fingerprints of wheat screened for *cytoplasmic male sterility* selective primers with 3- and 4-selective nucleotides. C4, C5, C6 and CR41 are the samples screened for *cytoplasmic male sterility / fertility*.

Lane1:	10 bp DNA ladder (Promega).
Lane 2:	Empty.
Lane 3-6:	Primer combination E-AGC; M-CAA.
Lane 7-10:	Primer combination E-AGCA; M-CAA.
Lane 11-14:	Primer combination E-AGCG; M-CAA.

Although the number of fragments did decrease, the percentage of polymorphic fragments did not increase in two of the four selective nucleotide primers (Table 2.10). The primer with the extra guanine generated more polymorphic fragments than the three selective nucleotide primers with almost a third less fragments in the fingerprint. The primer with the extra adenine generated less polymorphic

fragments than the three selective nucleotide primers but only about 20 % less fragments.

Table 2.10 Comparison of performance of primers with 3- and 4-selective nucleotide *Eco*R1 and *Mse*I M-CAC primers used for wheat *cytoplasmic male sterility*.

Primer	GC %	No. of fragments	No. of monomorphs	% Polymorphic DNA
E-AGC	50,0	51	45	11,7
E-AGCG	51,3	34	29	14,7
E-AGCA	48,7	40	36	10,0
E-ACAC	48,7	40	36	10,0
E-ACTC	48,7	49	45	8,1

2.4 DISCUSSION

Amplified fragment-length polymorphism (AFLP), a PCR-based fingerprinting technology is stringent, versatile, robust and quantitative (Zabeau and Vos, 1993). It involves the restriction of genomic DNA, followed by ligation of adapters complimentary to the restriction sites and selective PCR amplification of a subset of the adapted restriction fragments from many genomic sites (usually 50-100 fragments per reaction). These fragments are visualised on denaturing polyacrylamide gels through various methodologies and scored as dominant markers (Vos *et al.*, 1995). The availability of many different restriction enzymes and corresponding primer combinations provide a great deal of flexibility, enabling the direct manipulation of AFLP fragment generation for defined applications.

The power of AFLP methodology is based upon the molecular genetic variations that exist between closely related species, varieties or cultivars (Keim *et al.*, 1997). These variations in DNA sequence are exploited by the AFLP technology such that *fingerprints* of particular genotypes can be routinely generated. Depending on

genome characteristics, the resulting fingerprint may be complex, requiring a reduction of complexity; or may not produce suitable polymorphisms. Very homogeneous genomes may not be suitable for AFLP analysis (Rouppe van der Voort *et al*., 1997). The complexity of AFLP fingerprints is generally manipulated by adjusting the selective nucleotides or the CG content of the primer extensions (Thomas *et al*., 1995; Cervera *et al*., 1996a). It is known that many plant genomes are AT-rich, therefore by increasing the GC content of the primers may result in fewer bands (Keim *et al.*, 1997; Qi and Lindhout, 1997). In this study, maize *gamete sterility* had a downward trend in the amount of amplified loci with an increase in GC content of primers, while for wheat *eye spot resistance* an increase was observed in the amount of amplified loci with an increase in GC content of primers.

The AFLP fragments generated in this investigation ranged within the expected size range from 50 bp to about 900 bp (Vos *et al.,* 1995), providing sufficient usable fragments in the size range of 50 to 200 bp. The average number of fragments generated by a fingerprint ranged from 99 in the case of wheat *eyespot resistance* which was expected for a large genome (>1,5 X 10^{10} bp) to 42 for wheat *cytoplasmic male sterility* indicating the close relatedness of the genotypes. Maize fingerprints on the other hand, generated an intermediate number of fragments of about 60, which was in the expected range for a genome of a size in the order or 5 X 10^{8} to 6 X 10^{9} bp with three selective nucleotides (Cho *et al.*, 1996; Nakajima *et al.,* 1998).

The number of polymorphic loci generated by the different primer combinations varied greatly. 72,7 % of the primer combinations for maize *gamete sterility* produced polymorphic loci, while in wheat the primer combinations produced substantially less. 56,2 % for *eyespot resistance* and 17,2 % for *cytoplasmic male sterility*. These results indicate that the NIL of wheat are genetically different enough to produce a large percentage of polymorphic loci with the primer combination used. This was not the case for wheat *cytoplasmic male sterility* where the percentage of polymorphic loci was low, indicating a high level of homology between the genotypes. When the number of polymorphic loci was

scored according to the GC content of the primers, the number of polymorphic loci in maize increased with an increase in GC content. About 50 % polymorphic loci were produced with primers with 50 % CG content, indicating the polymorphic nature of the AT rich regions of the genome (Röder *et al.,* 1995). Overall for the wheat genotypes, the GC content did not change the amount of polymorphic loci significantly. In the case of *eyespot resistance* 30 % polymorphic loci were detected, while for *cytoplasmic male sterility* it was 12 %. This suggest that genome analysis of a complex genome (eg. polyploidy), such as for wheat, may require extensive technique research in order to find the most suitable combination of primers and selectable nucleotides to generate the most informative fingerprint.

CHAPTER 3

IDENTIFICATION AND CLONING OF MAIZE AND WHEAT TRAIT LINKED MARKERS

3.1. INTRODUCTION

The AFLP technique is a multilocus dominant marker system that provides simultaneous coverage of many loci in a single assay enabling the simultaneous visualisation of 60-100 loci per primer combination (Powell *et al.*, 1997). It is however difficult to distinguish between the different allelic products amplified from a single locus. The number of amplification products produced in one reaction is dependent on the primers that are used, and the species' genotype. In tomato (Thomas *et al.*, 1995), populus (Cervera *et al.*, 1996a) and sunflower (Hongtrakul *et al.*, 1997), it was found that AFLP technology produced a larger number of amplification products in each reaction, while in both barley (Qi and Lindhout, 1997) and soybean (Keim *et al.*, 1997) fewer amplification fragments were produced per primer combination.

The number of amplification products that are produced with a particular primer combination depends on a number of factors. In instances where the number of amplification products is too many and thus too difficult to score the number can be reduced by increasing the length of the primers. Hartl and Seefelder (1998) found that in hops cultivars two selective nucleotides produced too many fragments, while four produced too few. A combination of three selective nucleotides produced a scorable number of fragments. Another important factor to consider is the GC content of primers. Keim *et al.* (1997) found that the GC content of the primers affected the number of amplification products. The number of amplification products can therefore be tailored to produce fingerprints of varying complexity by changing the length and composition of the AFLP primer extensions (Qi and Lindhout, 1997).
Markers are selected by comparing fingerprints. Trait linked markers are identified as

co-migrating amplification products generated from a number of similar genotypes containing the trait of interest (Vos *et al*., 1995). There is however the possibility that co-migrating fragments are amplification products from different loci. The amplification products (using the same primers combinations) all have identical nucleotide compositions at the extremities of the fragments and although co-migrating fragments have similar mobility little is known about the core DNA sequence (Rouppe van der Voort *et al.*, 1997). It has been suggested that AFLP makers are highly informative because of amplification under stringent conditions, and it is unlikely that two amplification products of identical size arise due to mismatches in primer template annealing during amplification Becker *et al*. (1995). Furthermore, the estimation of mobility of amplification fragments in PAGE can be very accurate and the probability of coincidental co-migration can be as low as 0,03% (Rouppe van der Voort *et al.*, 1997). Qi *et al*. (1998) observed while constructing the molecular map of barley that these co-migrating fragments were alleles rather than loci and can therefore be converted into either probes or sequence tagged sites to identify their presence in segregating populations.

The conversion of fragments associated with a particular trait into diagnostic PCR-based markers relies upon efficient cloning and sequencing of purified DNA fragments (Mori and Fani, 1997). Once the amplification products have been sequenced, oligonucleotide primers of between 15-30 bp in length can be synthesised. These primers then make it possible to identify the presences or absence of a trait without prior sequence knowledge (Williams *et al*., 1996).

3.2 MATERIALS AND METHODS

All buffer and solution compositions are detailed in Appendix A.

3.2.1 Identification of polymorphic fragments.

AFLP fingerprints that were generated from the different genotypes of maize and wheat were evaluated for potential trait linked markers. Polymorphic fragments were identified by their presence in genotypes containing the trait of interest, and their

absence from fingerprints of genotypes that do not contain the trait of interest. These amplification products were identified and selected as possible markers.

3.2.2 Isolation of DNA fragments from polyacrylamide gel fingerprints

Two different methods were tested in isolating the DNA fragments from PAGE gels – a buffer based method and a dehydration method.

In the case of the buffer based method, the selected fragments were removed from the gel by using a scalpel blade. The segment of the gel, containing the fragment, was cut out and transferred to an eppendorf tube. Using a yellow tip, the slice of gel was crushed against the wall of the tube. Elution buffer (10 µl) was added to the tube and incubated at 37°C for four hours. The sample was centrifuged for one minute at 12 000 g at 4°C and the supernatant transferred to a new tube while avoiding transferring any fragments of polyacrylamide. An additional five µl (0.5 X the original volume of elution buffer) was added to the precipitate, vortexed briefly and centrifuged. The two supernatants were combined and placed on ice. Two volumes of ice-cold absolute ethanol were added and the tube stored on ice for 30 minutes. The DNA was recovered by centrifugation at 12 000 g for 10 min at 4°C. The DNA pellet was resuspended in 200 µl of 1 X TE buffer. The DNA was once again precipitated by the addition of 25 µl of three molar sodium acetate (pH 5,2) and two volumes of absolute ethanol. The pellet was rinsed in 70 % ethanol, and redissolved in 10 µl 1 X TE.

The method described by Chalhoub *et al.* (1997) to recover AFLP fragments from PAGE gels by dehydration was also tested. This protocol avoids the use of any buffers and laborious cleaning of the DNA. Five microlitres of sterile distilled water was placed and left over the silver stained DNA fragment/s of interest to rehydrate the PAGE gel for 30 min. The water was removed with a sterile tip and placed in a PCR tube to be amplified later.

3.2.3 PCR amplification of isolated AFLP fragments

In order to determine whether the identified AFLP fragment were amplification products of a single locus, the DNA isolated from a band was used as a template for PCR amplification. This was done so that the amplification products could be used to determine the presence of a single template. In a PCR tube on ice, two µl extracted DNA, 0,5 µl of *Eco*RI primer, 4,5 µl *Mse*I primer, two µl 10 X AFLP buffer and 0,4 µl five U/µl GibcoBRL *Taq* DNA polymerase were added and made up to a final volume of 20 µl with ddH_2O. After gentle mixing, the tube was centrifuged to collect the reaction components which were then overlaid with 2 drops of silicone oil. The DNA fragment was amplified in a Hybaid Omnigene TR3 CM220 thermocycler by 23 cycles of 30 sec at 94°C, 1 minute at 56°C, and 1 minute at 72°C, followed by one cycle of 7 minutes at 72°C. The whole amplified sample was loaded on a 3 % agarose gel containing 1 µg/ml EtBr to separate the fragments.

3.2.4 Purification of amplified fragments

The Magic PCR Preps™ kit (Promega, Cat no.#A7190) was used to purify the amplified PCR products separated on agarose. To the excised agarose gel slice, 100µl of 1 X TE buffer was added and the gel slice melted at 100°C. Ten µl Magic PCR Preps™ resin was then added to the melted solution. Contaminants such as dNTP's and amplification primers, were separated from the DNA through the selective binding of the DNA to the Magic PCR Preps™ resin, which in turn binds directly to the column. The melted solution was added to the column, and the column centrifuged in a bench top centrifuge for one minute at maximum speed. Thereafter, the column was washed by adding 100 µl of 70 % ethanol and then centrifuged in a bench top centrifuge at maximum speed for one minute. The DNA fragments were then eluted by adding 20µl of 1 X TE buffer to the column and again centrifuging for one minute.

3.2.5 Preparation of cloning vector

Large-scale extraction of pSK cloning vector

An upscale version of the Ish-Horowicz and Burke method (1981) was used to extract the cloning vector plasmid, pSK. *E. coli* strain DH5α containing the pSK vector was streaked out on a Luria agar plate to produce single colonies. A single bacterial colony was picked from the Luria agar plate containing 10 µg/ml Ampicillin and grown overnight in 400 ml Luria broth containing 100 mg/ml Amplicillin. The cells were collected by centrifugation at 5000 rpm for five minutes in a Beckman centrifuge and resuspended in five ml of TSE buffer and transferred to a centrifuge tube. The cells were collected by further centrifugation at 5 000 rpm for five minutes in a Beckman centrifuge and the remaining liquid drained from the pellet with a pipette and left to air dry. The dried pellet was resuspended in three ml of solution 1 (composition provided in appendix A) and left at room temperature for 10 minutes to disrupt the cells. To the lysed cells, six ml of solution 2 (composition provided in appendix A) was added and immediately placed on ice for a maximum of five minutes, after which 4,5 ml of precooled solution 3 (composition provided in appendix A) was added, mixed well and placed on ice for a further five minutes. The solution was then centrifuged at 12 000 rpm for 15 minutes to precipitate the protein, SDS and chromosomal DNA. The supernatant was transferred to plastic tube, 13,5 ml isopropanol was added to the supernatant and left at -70°C for 30 minutes to precipitate the plasmid DNA. After centrifugation at 10 000 rpm for 10 minutes to collect the plasmid DNA, five ml of 1 X TE buffer was added to resuspended the plasmid DNA.

Purification of pSK cloning vector

Five ml of TE suspended DNA was purified by the addition of five gram of CsCl and 200 ml of 10 mg/ml Ethidium bromide. The solution was cleaned by centrifugation at 10 000 rpm for 10 minutes. The clear, red solution was transferred to a Beckmann quick-seal ultracentrifuge tube with a disposable syringe fitted with a large-gauge needle. The tube was sealed, placed in a Ti65 rotor and centrifuged at 55 000 rpm overnight at 4°C. After centrifugation, the tubes were removed from the rotor and placed in front of an ultra violet light to visualise the DNA fragment. Two fragments

were identified, at the top the open circle conformation of the pSK plasmid, and at the bottom the supercoiled conformation of the plasmid. The bottom DNA fragment was extracted by carefully inserting a syringe above and below the band of interest and collected in a 1,5ml eppendorf tube. To the solution, an equal amount (one ml) of salt saturated isopropanol was added and the tube inverted a few time to extract the EtBr. The solution was allowed to separate. The top pink solution was then removed. These steps, from the addition of isopropanol, were repeated until the solution became clear. The CsCl in the solution was removed through dialysis for three hours in dialysing tubing in 1 X TE buffer at 4°C. The tubing was then placed in a fresh solution of 1 X TE buffer and dialysed further overnight. The cleared plasmid solution was removed from the tubing and placed in a 1,5ml eppendorf tube and the DNA concentration determined by scanning in a UV spectrophotometer between 220 nm and 320 nm wavelengths.

Restriction digestion of pSK cloning vector

The plasmid DNA concentration was adjusted to a final concentration of 100 ng/µl. In a total volume of 20 µl, 10 µl of DNA, two µl of buffer B (composition provided in appendix A) and 0,1 µl of *Eco*RV enzyme (3 units) were added to an eppendorf tube on ice. The solutions were mixed gently, collected by brief centrifugation and incubated for two hours at 37°C followed by incubation for 15 min at 70°C to inactivate the restriction enzyme. The tube was placed on ice and the reaction collected by brief centrifugation. To the reaction, 20 µl of sodium acetate (pH 5,2) and 200 µl of 100 % isopropanol were added. The tube was left for one h at -20°C to precipitate the DNA after which the solution was centrifuged for 15 minutes at 12 000 rpm in a refrigerated centrifuge to collect the plasmid DNA. The pellet was washed by adding 500 µl of 70 % ethanol and centrifuged for 15 minutes at 12 000 rpm in a refrigerated centrifuge. The pellet was dried in a speedy vac dryer and dissolved in 20 µl of 1 X TE buffer.

Production of T-tailed pSK cloning vector

Taq polymerase has an adenosine transferase activity that adds an adenine to the 3' end of the PCR product. This activity can be used to ligate the PCR product to a T-

tailed vector. A T-tailed vector was prepared by mixing together 0,4 µl of 0,1 µM dTTP, 10 µl of 50 ng/µl *Eco*RV digested pSK, 0,1 µl of 5 U/µl *Taq* DNA polymerase and 1,5 µl of *Taq* buffer in an eppendorf tube on ice. The solution was made up to a final volume of 15 µl with ddH_2O. The tube was mixed gently, the reaction collected by brief centrifugation and incubated for two hours at 70°C. The tube was placed on ice and the reaction again collected by brief centrifugation. The quality of the DNA was determined by loading the whole reaction on a 0,8 % agarose gel containing 1 µg/ml EtBr.

Extraction of T-tailed pSK cloning DNA vector from agarose

The T-tailed *Eco*RV DNA fragments that were separated by gel electrophoresis on a 0,8% agarose gel were cut with a scalpel from the gel, placed in an eppendorf tube and purified with the GENECLEAN II kit (Bio 101, Inc., Cat # 1001-400). The gel slice was weighed and three NaI added per µg of agarose. After incubation at 55°C for five minutes to melt the agarose and release the DNA, five µl glass milk suspension was added. The solution was incubated for five minutes on ice and then centrifuged for five minutes. The supernatant was removed by pipeting, and the pellet was washed three times with 500 µl New Wash buffer. After the third wash, the pellet was compacted with centrifugation for one minute, and the excess New Wash buffer removed. The pellet was resuspend in 10 µl 1 X TE and incubated at 55°C for 15 minutes to detach the DNA from the glass milk matrix. The solution was centrifuged for one minute and the supernatant collected in an eppendorf tube. DNA concentration was estimated by loading three µl on a three percent agarose gel containing one µg/ml Ethidium bromide and then comparing it to Lambda DNA with a concentration of 100 ng.

3.2.6 Cloning of AFLP fragment

Ligation of amplification products

A volume of 15 µl was prepared by mixing together in an eppendorf 11,25 µl of AFLP amplified DNA, 1,5 µl ligase buffer, 0,75 µl 10 mM ATP, 0,5 µl 100 ng/µl T-tailed pSK, and 1 µl ligase. The solution was mixed gently, collected by brief centrifugation and incubated for one hour at 16°C. The mixture was incubated for 15 min at 70°C to inactivate the enzyme. The tubes were placed on ice for 10 minutes and the reaction collected by brief centrifugation.

Preparation of E. coli bacterial competent cells

An overnight culture of *E. coli* DH5α strain was grown and then streaked out on a Luria agar plate that did not contain any antibiotics. One colony of DH5α was picked and grown in 10 ml of LB at 37°C while shaking. 400 µl overnight grown culture of DH5α was added to 200 ml pre-warmed (37°C), sterilised Luria broth (LB) containing 0,1 % (200 µg) glucose and grown to an OD_{600} of 0,2. The culture was then cooled immediately on ice for five minutes. The cells were harvested in a centrifuge using a GSA rotor at 4 000 rpm for five minutes. The supernatant was poured off and the bacterial cell pellet resuspended in 100 ml ice cold 0,1 M $CaCl_2$. The cell suspension was placed on ice for 20 minutes and then centrifuged in a centrifuge using a GSA rotor at 3 000 rpm for five minutes. The supernatant was poured off and the bacterial cell pellet resuspended in two ml of 0,1M $CaCl_2$. The cells were stored on ice at 4°C and used immediately for subcloning.

Transformation of competent cells

To an eppendorf tube on ice, 100 µl competent cells and five µl ligation mix, containing the amplification products ligated to T-tailed pSK, were added, gently mixed and left on ice for 30 minutes. The cells were heat shocked by placing the tube in a water bath at 42°C for 90 seconds. The tube was then placed on ice for two minutes to cool down. Pre-warmed (37°C), sterilised LB (800 µl) was added to the cell mixture and incubated for one hour at 37°C. The cells were initially grown in Luria

broth for one hour to induce the ampicillin resistance and then plated on Luria agar plates containing 100 µg/ml ampicillin coated with 50 µl 20 mg/ml X-gal and 10 µl 23,4 mg/ml IPTG to select cells that were resistant to ampicillin. The plates were then sealed in a plastic bag and incubated overnight at 37°C.

Identification of bacterial colonies containing recombinant plasmids

Bacterial colonies that contained plasmids with AFLP fragment inserts were identified through α-complementation in which both the vector and the recipient cell play a roll. The vector, pSK, contains a short segment of a regulatory sequence of *E. coli* that codes for a part of the β-galactosidase gene (*lacZ*). Embedded in this coding region is the multiple cloning site that does not disturb the reading frame but results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of β-galactosidase. The *E. coli* strain used for the transformation, DH5α, codes for the carboxyl-terminal portion of β-galactosidase. Although neither the host-encoded nor the plasmid-encoded fragments are themselves active, they can associate to form an active enzyme protein. The Lac^+ bacteria that result from α-complementation are easily recognised because they form blue colonies in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). However, insertion of the AFLP fragment into the multiple cloning site produces a recombinant plasmid that almost invariably results in the production of an amino-terminal fragment that is not capable of α-complementation and therefore produces white colonies which are easily distinguished from the non recombinant white colonies.

Plasmid DNA extraction

A downscaled miniprep version of the Ish-Horowicz and Burke method (1981) was used to extract the recombinant pSK vectors containing an AFLP insert from white colonies. A single white bacterial colony was picked from the plate and grown overnight in five ml Luria broth containing 100 mg/ml ampicillin. The cells were then collected by adding 1,5 ml of bacterial culture to an eppendorf tube, and then centrifuged at 12 000 rpm for one minute in a bench centrifuge. The supernatant was

removed and the cell pellet resuspended in one ml of TSE buffer. The cells were precipitated by centrifugation at 12 000 rpm for one minute in a bench centrifuge. The remaining liquid was removed from the cells with a pipette and left to dry for 5 minutes. The dried pellet was resuspended in 100 µl of solution 1 (composition provided in appendix A) and left at room temperature for 10 minutes. To the lysed cells, 200 µl of solution 2 (composition provided in appendix A) were added and the tube placed on ice for a maximum of five min. 150 µl of pre-cooled solution 3 (composition provided in appendix A) was added to the tube, mixed well and placed on ice for a further five minutes. The solution was centrifuged at 12 000 rpm for 15 minutes to precipitate the protein, SDS and chromosomal DNA. To the supernatant, 45 µl of three molar sodium acetate and 500 µl of isopropanol were added and left at -70°C for 30 minutes to precipitate the plasmid DNA. The solution was centrifuged at 12 000 rpm for 15 minutes to collect the plasmid DNA. The supernatant was removed and 500 µl 70 % ethanol added to the pellet and then centrifuged at 12 000 rpm for 10 minutes. All the ethanol was removed from the pellet and 20 µl 1 X TE buffer added.

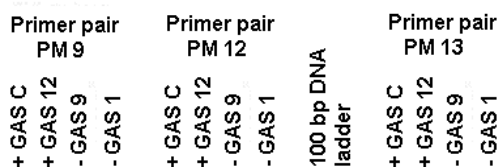
Restriction enzyme digestion

To confirm that the fragment of the correct size was ligated into the vector, the extracted plasmid was cut with the restriction enzyme *Pvu*II. This enzyme was used because it restricts pSK on either side of the multiple cloning site, excising a fragment of 445 bp. For comparison purposes, non-recombinant pSK was also digested with *Pvu*II as a control. The sizes of the fragments were determined by comparing them with known sizes of DNA fragments of Lambda DNA digested with restriction enzyme *Pst*1. To each of the two types of plasmid extracts, kept on ice, 2,5 µl buffer M and 0,1 µl of *Pvu*II enzyme was added and made up to a total volume of 25 µl with ddH_2O. The solutions were gently mixed, collected by brief centrifugation and incubated for 2 h at 37°C. The solutions were then incubated for 15 min at 70°C to inactivate the restriction enzymes. The tubes were placed on ice and the reaction collected by brief centrifugation. The restricted plasmids were run on a 0,8 % gel together with a molecular weight marker (Lambda DNA digested with *Pst*I enzyme).

3.3 RESULTS

3.3.1 Identification of polymorphic AFLP fragments

The fingerprints of all the traits showed the presence of polymorphic fragments. In figure 3.1, fragment 1 is coupled to the trait of interest (coupling) and fragment 2 is present in the genotypes where the trait of interest is absent, it is in a state of repulsion. Only fragments in coupling were considered as possible markers since they could be directly linked to the trait of interest.



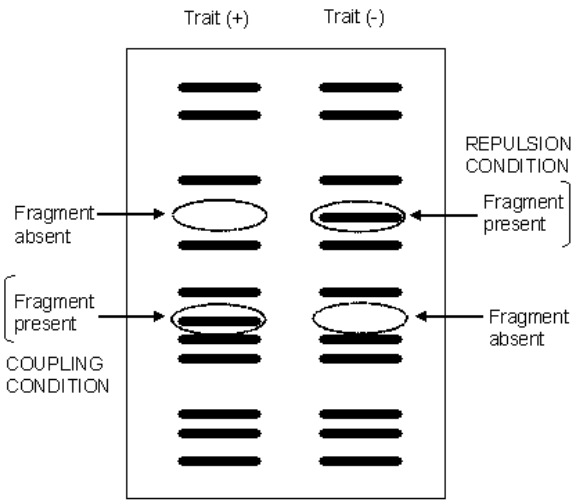


Figure 3.1 AFLP fingerprint of maize *gamete sterility* generated with primer combinations PM9 (E-ACA; M-CTG), PM12 (E-ACT; M-CAG) and PM13 (E-ACT; M-CAT). The numbers 1 (marker 10M9A) and 2 indicates the polymorphic fragments.

In maize screened for *gamete sterility*, 13 out of 22 primer combinations tested produced polymorphic fragments that could be selected for further investigation (Table 3.1). Out of 40 identified polymorphic fragments, 17 were in coupling and marked as potential markers that could be linked to *gamete sterility* (Figure 3.2).

Table 3.1 Primer combinations that identified polymorphisms and number of polymorphic fragments identified for maize *gamete sterility*.

Trait	Genotypes	Primer combination	Number of polymorphic fragments		Selected polymorphic fragment
			C	R	
Maize *Gamete sterility*	GAS C*	PM1 (E-AAC; M-CAG)	1	1	12M1A
	GAS 12*	PM3 (E-AAC; M-CTG)	2	2	
	GAS 9	PM4 (E-AAC; M-CTG)	2	3	
	GAS 1	PM6 (E-ACA; M-CAC)	3	0	
		PM7 (E-ACA; M-CAT)	0	2	
		PM9 (E-ACA; M-CTG)	1	2	10M9A
		PM10 (E-ACA; M-CTT)	1	1	
		PM15 (E-ACT; M-CTC)	0	3	
		PM16 (E-AGG; M-CAC)	2	3	10M16C
		PM18 (E-AGG; M-CTA)	2	0	
		PM19 (E-AGG; M-CTG)	0	3	
		PM20 (E-AGC; M-CTG)	0	1	
		PM21 (E-AGC; M-CAT)	3	1	

* Positive genotypes
R Repulsion
C Coupling

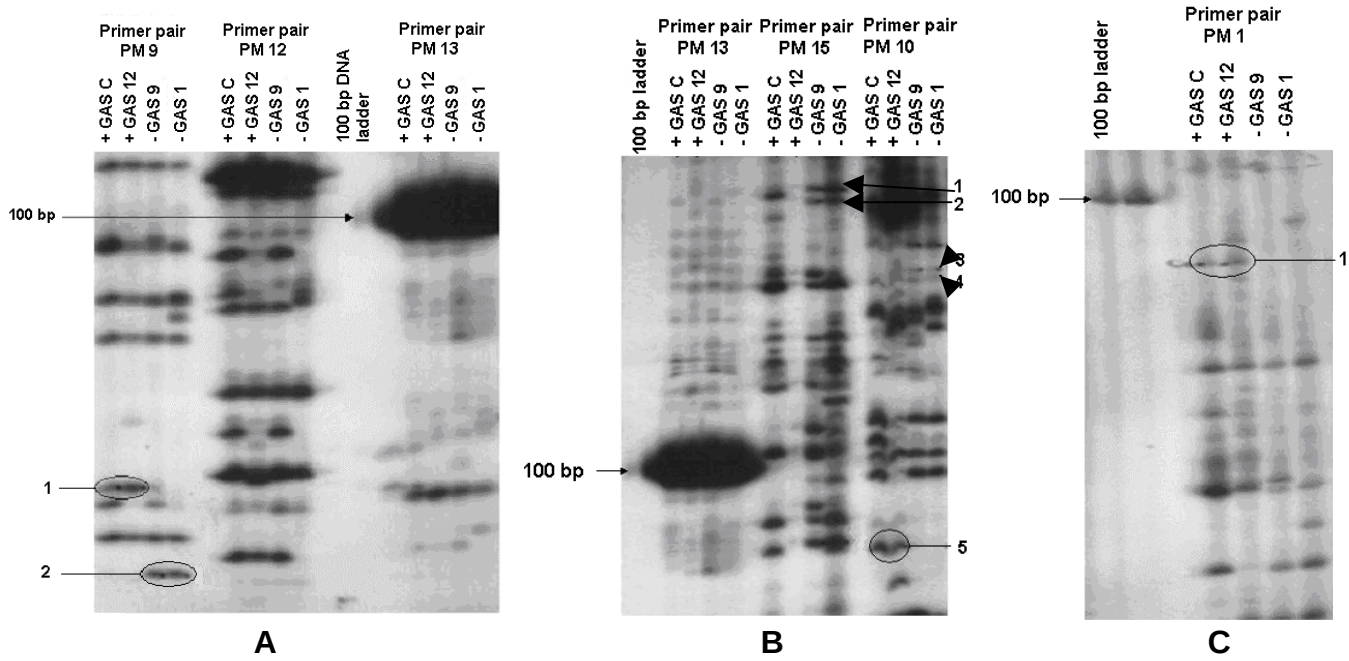


Figure 3.2 AFLP fingerprints of maize *gamete sterility.* A: Generated with primer combinations PM9 (E-ACA; M-CTG), PM12 (E-ACT; M-CAG) and PM13 (E-ACT; M-CAT). The numbers 1 (marker 10M9A) and 2 indicates the polymorphic fragments. B: Generated with primer combinations PM13 (E-ACT; M-CAT), PM15 (E-ACT; M-CTC) and PM16 (E-AGG; M-CAC). The numbers 1-5 indicate the polymorphic fragments. Fragment 5 is the marker 10M16C. C: Generated with primer combinations PM1 (E-AAC; M-CAG). Number 1 indicates the polymorphic fragment. Fragment 1 is the marker 12M1A.

It was noticed that the polymorphic loci occurred at a greater frequency in the fingerprints generated with primers with a high GC content (Table 3.2). It should however be mentioned that this could be because of biased sampling with only certain primer combinations.

Table 3.2 Comparison of the amount of polymorphic loci obtained with AFLP primer combinations with different GC contents for maize screened for *gamete sterility*.

% Primer GC content	Lowest number of polymorphic fragments	Highest number of polymorphic fragments	Average number of polymorphic fragments
44,7	2	2	2
47,4	2	5	3,75
50	1	5	3

In wheat *eye spot resistance*, 18 out of 49 primer combinations tested, produced polymorphic fragments that could be selected for further investigation (Table 3.3). Out of 55 identified polymorphic fragments, 32 were in coupling and marked as potential markers that could be linked to wheat *eye spot resistance*.

In wheat *eye spot resistance* a lower frequency of polymorphic fragments in the fingerprints generated with primers with a high GC content was observed (Table 3.4).

Table 3.3 Primer combinations and number of polymorphic fragments identified for wheat *eye spot resistance.*

Trait	Genotypes	Primer combination	Number of polymorphic fragments		Selected polymorphic fragment
			C	R	
Wheat *Eye spot Resistance*	E9*	PE 3 (E-AAC; M-CAG)	0	1	
	E10	PE9 (E-ACA; M-CAA)	2	1	
	E12	PE10 (E-ACA; M-CAG)	5	2	
	E13*	PE11 (E-ACA; M-CTC)	5	0	
		PE12 (E-ACA; M-CAC)	1	2	
		PE13 (E-ACA; M-CAT)	1	0	
		PE17 (E-ACT; M-CAA)	5	4	
		PE19 (E-ACT; M-CAG)	1	1	
		PE23 (E-ACT; M-CTG)	2	0	
		PE27 (E-AGG; M-CAG)	2	0	
		PE31 (E-AGG; M-CTG)	1	2	K311
		PE32 (E-AGG; M-CTT)	0	2	
		PE33 (E-AGC; M-CAA)	1	0	
		PE35 (E-AGC; M-CAG)	0	1	
		PE37 (E-AGC; M-CTA)	3	2	
		PE38 (E-AGC; M-CTC)	0	1	
		PE43 (E-ACC; M-CAG)	2	1	
		PE64 (E-ACG; M-CTT)	1	3	

* Positive genotypes
R Repulsion
C Coupling

Table 3.4 Comparison of the number of polymorphic loci obtained with AFLP primer combinations with different GC contents for wheat screened for the fungal resistance trait.

% GC content	Lowest number of polymorphic fragments	Highest number of polymorphic fragments	Average number of polymorphic fragments
44,7	1	9	4,3
47,4	1	7	3,3
50	1	3	2

In wheat *cytoplasmic male sterility* 6 out of 30 primer combinations tested produced polymorphic fragments that could be selected for further investigation (Table 3.5). All 6 of the identified polymorphic fragments were in repulsion. It was therefore decided to use these fragments that were in a repulsion state with wheat *cytoplasmic male sterility* as putative markers (Figure 3.4).

Table 3.5 Primer combinations and amounts of polymorphic fragments isolated for wheat *cytoplasmic male sterility*.

Trait	Genotypes	Primer combination	Number of polymorphic fragments		Selected polymorphic fragments
			C	R	
Wheat *Cytoplasmic male sterility*	C4*	PC12C (E-ACAC; M-CAG)	0	1	
	C5*	PC18C (E-ACTC; M-CAC)	0	1	
	C6	PC35 (E-AGC; M-CAG)	0	1	K35
	CR41	PC35A (E-AGCA; M-CAG)	0	1	K35AMS1
		PC35G (E-AGCG; M-CAG)	0	1	
		PC36 (E-AGC; M-CAT)	0	1	K36

* Positive genotypes
R Repulsion
C Coupling

Table 3.6 Comparison of the number of polymorphic loci obtained with AFLP primer combinations with different GC contents for wheat *cytoplasmic male sterility*.

% GC content	Lowest number of polymorphic fragments	Highest number of polymorphic fragments	Average number of polymorphic fragments
47,4	1	1	1
48,7	1	1	1
50	1	1	1
51,3	1	1	1

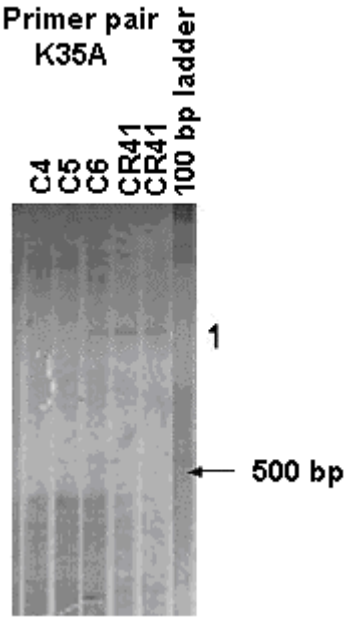


Figure 3.3 AFLP fingerprint of wheat *cytoplasmic male sterility* generated with primer combination PC35A (E-AGCA; M-CAG). Number 1 indicates the polymorphic fragment identified in restore CMS (CR41).

It was observed that the number of polymorphic loci did not increase or decrease with an increase in GC content of the primers. The biased sampling with only certain primer combinations, may have led to the frequency of all the polymorphic loci to be the same.

3.3.2 Establishment of single locus amplification AFLP fragments

DNA concentration of isolated DNA from PAGE gels could not be determined by agarose gel electrophoresis. The DNA was amplified with a modified reaction and PCR conditions for pre-amplification to determine if any DNA was present. Only AFLP fragments resulting from the amplification of a single locus were cloned and tested as putative markers. In order to establish this, the selected AFLP fragments were subjected to PCR amplification and electrophoresis. AFLP DNA extracted from polyacrylamide gels using the high salt extraction method gave the best PCR amplification results.

Optimisation of amplification of extracted DNA fragment

DNA concentration had the greatest effect on the amplification product. The best conditions were obtained by adding no more than two µl of the isolated AFLP DNA in a 15 µl reaction. Higher concentrations (5 µl) produced smears, while lower concentrations (one µl) produced too little amplified product.

An increase in $MgCl_2$ concentration in the PCR reaction had no effect on the yield of the amplified product. It was found that a concentration of 15 mM was sufficient to produce specific amplification without any accompanying unspecific amplification. The PCR product yield increased with a proportional increase of reaction volumes, however two µl of isolated AFLP DNA in 15 µl reaction volume with 15mM $MgCl_2$ produced ample amplification product, in the order of ca. 1,6 ng/µl (Figure 3.4).

The following fragments (Table 3.7) were chosen for cloning and testing.

Table 3.7 Fragments selected to be used in further manipulations.

Trait	Fragment	Primer combination	Size
Maize gamete sterility	10M9A	E-ACA M-CTG	ca. 80bp
	10M16C	E-AGG M-CAC	ca. 100bp
	12M1A	E-AAC M-CAG	ca. 100bp
Wheat eye spot resistance	K311	E-AGG M-CTG	ca. 100bp
Wheat cytoplasmic male sterility	K35	E-AGC M-CAG	ca. 250bp
	K35AMS1	E-AGCA M-CAG	ca. 800bp
	K36	E-AGC M-CAT	ca. 200bp

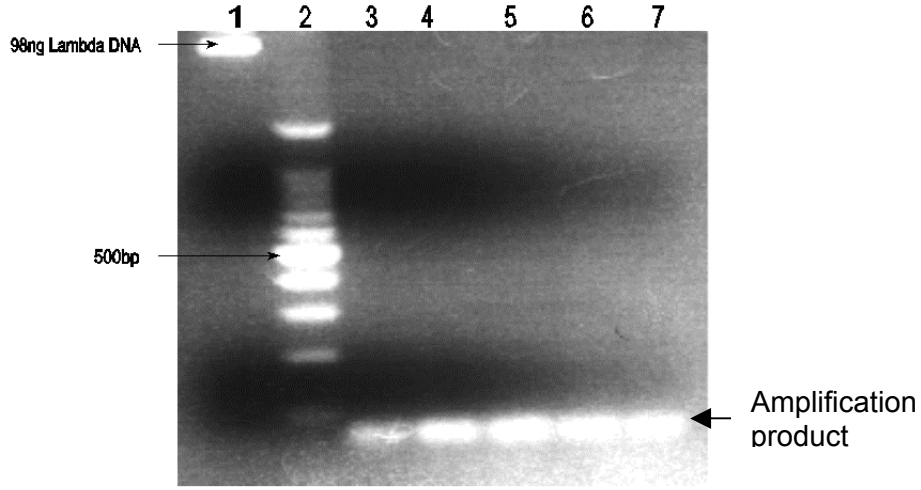


Figure 3.4 A 3% agarose gel containing the re-amplified fragment 10M9A in different reaction volumes (lanes 3 – 7).

Lane 1: 98 ng of Lamda DNA.

Lane 2: 100 bp DNA ladder.

Lane 3: Re-amplified 10M9A obtained from a reaction volume of 15 µl.

Lane 4: Re-amplified 10M9A obtained from a reaction volume of 25 µl.

Lane 5: Re-amplified 10M9A obtained from a reaction volume 50 µl.

Lane 6: Re-amplified 10M9A obtained from a reaction volume 75 µl.

Lane 7: Re-amplified 10M9A obtained from a reaction volume 100 µl.

3.3.3 Validation of cloned fragments

The seven chosen AFLP polymorphic markers (10M9A, 10M16C, 12M1A, K311, K35, K35AMS1, K36) were ligated to the T-tailed *Eco*RV site of pSK. After ligation competent *E. coli* strain DH5α cells were transformed and the recombinant colonies were identified (white colonies). The proportion of blue (non recombinant colonies) to white colonies (recombinant colonies) occurred in a ratio of about 1 : 1. The white colonies were picked from the Luria agar plates, the presence of the vector validated by restriction digestion with *Pvu*II to release the insert and the digested products separated on an agarose gel. The fragment sizes could be estimated by comparison to *Pvu*II enzyme digestion of native pSK. The native pSK separated into two fragments on the agarose gel, one of 2 514 bp and the other 445 bp, which contained the multiple cloning site. Any inserts would therefore be larger than 445bp. Figure 3.5 shows native and recombinant digestion products on a three percent agarose gel.

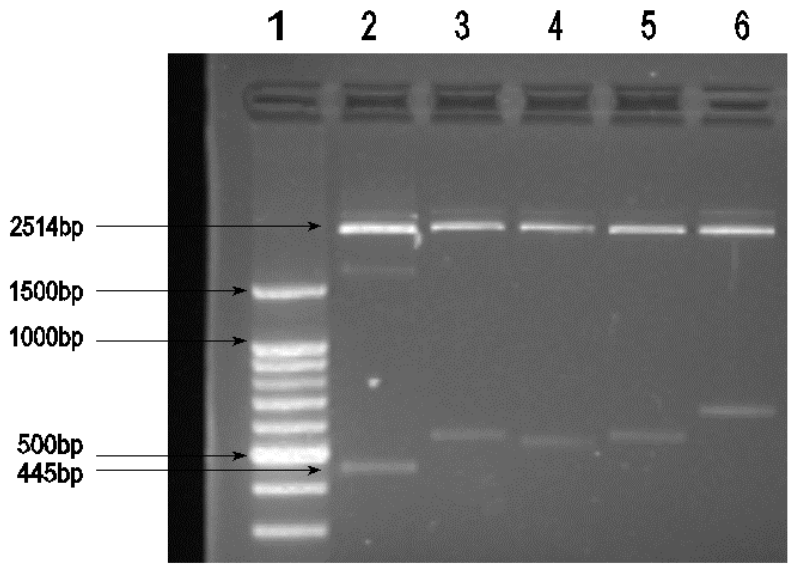


Figure 3.5 Three percent agarose gel containing recombinant plasmids restricted with *Pvu*II to estimate the sizes of the inserts.

Lane 1: Lambda DNA restricted with *Pst*I

Lane 2: pSK restricted with *Pvu*II

Lane 3: Recombinant plasmid 12M1A restricted with *Pvu*II

Lane 4: Recombinant plasmid 10M9A restricted with *Pvu*II

Lane 5: Recombinant plasmid 10M16C restricted with *Pvu*II

Lane 6: Recombinant plasmid K36 restricted with *Pvu*II

The agarose gel separation of digestion products was used to estimate the size of the AFLP fragments (Table 3.8).

Table 3.8 Fragment sizes of the restricted recombinant plasmids.

Trait	Fragment	Size of *Pvul*l enzyme restricted fragments
Maize gamete sterility	10M9A	2514bp + 525bp
	10M16C	2514bp + 545bp
	12M1A	2514bp + 545bp
Wheat eye spot resistance	K311	2514bp + 545bp
Wheat cytoplasmic male sterility	K35	2514bp + 695bp
	K35AMS1	2514bp + 1245bp
	K36	2514bp + 645bp

3.4 Discussion

Polymorphic markers linked to maize and wheat traits were successfully generated and evaluated. It was interesting to note that the number of polymorphic fragments generated with the AFLP technology was much greater in maize than in wheat. In maize *gamete sterility*, 81 % of the generated fragments were polymorphic, while for wheat *eye spot resistance* only 67 % were polymorphic. The most probable explanation for this difference may lie in the genome composition of the two species.

Maize, a diploid has 10 chromosomes pairs (x = 10) while wheat, a hexaploid, consists of three different genomes (AABBDD, 2n = 42) (Poehlman, 1987). The formation of such a successful hexaploid involved hybridisation events in the evolution of the species, which suggests extensive similarity (homoeolology) between the genomes (A, B and D) and repetition of loci over the three genomes (Van Deynze *et al.*, 1995). Although authors describe the genome of wheat as large, in reality there are three genomes (A, B and D) that are for the most, replications of one another. On the other hand, little is known about the developmental history of the wheat genotypes

CHAPTER 4

CHARACTERISATION OF PUTATIVE MARKERS AND PRIMER DESIGN

4.1 INTRODUCTION

Three putative markers were identified for maize *gamete sterility*, one for wheat *eye spot resistance*, and three for wheat *cytoplasmic male sterility*. In order to evaluate the usefulness of using these markers to identify the presence of the traits in other individuals, it was necessary to characterise the fragments and to design specific primers. This was accomplished by firstly converting the fragments to sequence characterised amplified regions (SCARs).

A SCAR is a PCR based marker of a specific nucleic acid sequence of an allele at a specific locus of a specific genotype (Naqvi and Chattoo, 1996). These regions are identified by primers designed specifically for the ends of the region, which are amplified under specific amplification conditions (Akagi *et al.*, 1996; Williams *et al*., 1996). In this investigation SCARs were prepared and evaluated as potential DNA markers for maize *gamete sterility*, wheat *eye spot resistance* and wheat *cytoplasmic male sterility*. For wheat *cytoplasmic male sterility*, none of the polymorphic fragments were in a state of coupling with the trait. The SCARs were therefore identified in the genotypes not displaying *cytoplasmic male sterility*.

Two approaches were followed to produce the SCAR primers; one that included the restriction enzyme sequence that produced the genomic fragments at the beginning of the AFLP method, and one that excluded the restriction enzyme sequence. Once suitable primers were designed, the fragment was then amplified using genomic DNA of an individual known to contain the sequence homologous

to the specific primers and thereafter the amplification products separated in an agarose gel. The development of SCARs therefore eliminates working with harmful chemicals such as acrylamide in PAGE. It also simplifies the identification of individuals containing the marker fragment.

4.2 MATERIALS AND METHODS

4.2.1 Sequencing of putative markers

The seven selected AFLP fragments, cloned into the multiple cloning site of pSK of *Eco*RV, were flanked by two primer-binding sites, which made it possible to sequence any ligated fragment. The seven AFLP fragments were sequenced according to the Sanger sequencing procedure (Sanger *et al*., 1977) by the University of Cape Town sequencing services using primers that bound to these primer-binding sites, and thus producing sequenced characterised regions (SCARs).

4.2.2 Primer design and primer testing

The sequence information was then used to design specific primers to amplify each of the SCARs. Since each SCAR originated from an AFLP fragment generated through the use of *Eco*RI and *Mse*I enzyme restriction, the ends of the SCARs contained these sequences and could either be included or excluded when designing primers. Where the sequence of the selective primers and restriction enzyme sequences could be identified, the primers were designed around the 3' end of the selective primer sequence to include the restriction enzyme sequence. In instances where it was difficult to identify the precise sequence of the selective primer, the primer sequence was designed by choosing sequences about 15 bp (an average of the length of the selective primer of both

the *Eco*RI and *Mse*I primers that would exclude the *Eco*RI and *Mse*I restriction sites) from the ligation junction of the plasmid and insertion fragment.

The forward primer (at the 5' end of the sequence) was designed to have the same sequence as the sequenced fragment. The reverse primer (at the 3' end of the sequence) was designed to have the complementary sequence of the sequenced fragment to allow for amplification during the PCR. In instances where the restriction enzyme sequence could be identified, the forward primer was designed to identify the *Eco*RI sequence, and the reverse primer to identify the *Mse*I sequence.

The wheat SCAR SK35, associated with wheat *cytoplasmic male sterility,* was used to determine which of the two primer design options produced the better result by attempting amplification using genomic DNA of the four wheat *cytoplasmic male sterile and restorer* genotypes C4, C5, C6 and CR41. The fragments were amplified by adding to a PCR tube on ice, two µl of extracted genomic DNA (20 ng), 2,5 µL forward- and 2,5 µL reverse primer (0,25 µM each), 2,5 µl buffer, 2,5 µl $MgCl_2$ (2,5 mM) and 0,2 µl five U/µl GibcoBRL *Taq* DNA polymerase and the final volume made up to 25 µl with ddH_2O. After gentle mixing, the tube was centrifuged to collect the reaction components, which were then overlaid with two drops of mineral oil.

Amplification temperatures are specific for each primer set and needs to be determined for each primer set prior to amplification. The optimal annealing temperature (Tm) of the SK35 primer set was calculated according to the following formula.

$$Tm_{combined} = \frac{(Tm_1 + Tm_2)}{2}$$

Where Tm_1 = 2 X (total number of A and T nucleotides) + 4 X (total number of G and C nucleotides) of the forward primer.

Tm_2 = 2 X (total number of A and T nucleotides) + 4 X (total number of G and C nucleotides) of the reverse primer.

The DNA fragment was amplified in a Hybaid Omnigene TR3 CM220 thermocycler with an initial denaturing step of five minutes at 94°C, and then 30 cycles of one minute at 94°C, one minute at the specific primer combination annealing temperature (Tm), and one minute at 72°C, followed by one cycle of four minutes at 72°C. The total sample was loaded on a 3 % agarose gel containing 1 µg/ml ethidium bromide.

The results obtained from SCAR SK35 indicated that primers that included the restriction sites produced better results (presented in results), therefore, all other primer sets were designed to include the restriction sites.

4.2.3 Validation of SCAR primers

The effectiveness of the SCAR primers required validation by the amplification of the SCAR sequences of the genotypes containing the sequences and then determining whether the amplification products were of the expected sizes. The

reaction conditions and PCR programme for most of the reactions were the same as those used for SCAR SK35 (section 4.2.2). However, for some of the wheat SCARs, an alternate approach was employed, since the *Eco*RI and *Mse*I restriction enzyme sites could not be identified in all the AFLP fragments. Therefore, sequences at 15 bp from the plasmid-fragment-insertion junction, were selected, so as to exclude the 15 bp region that may have included the sequence containing the selective primer sequence.

4.3 RESULTS

4.3.1 Sequences of selected AFLP fragments

All seven selected AFLP fragments were sequenced and converted to SCARs and ranged in size from 80 bp to 800 bp (Table 4.1).

It was not possible to identify the selective amplification primers and restriction site in all seven of the AFLP SCAR fragments. These sequences at both the 3' end and 5' end could be identified in five of the SCARs, namely S10M16C, S10M9A, S12M1A, SK311, and SK35 while the complementary sequences could be identified in SCAR SK35AMS1 and SK36 (Table 4.1).

Table 4.1 SCAR sequences and sizes. Sequences are between the two plasmid-insertion-fragment junctions.

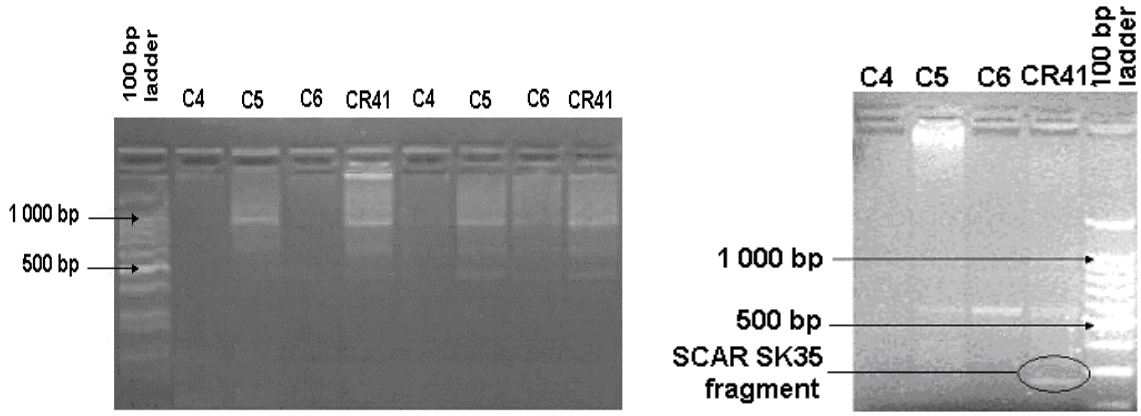
Trait	SCAR	Sequence	Size (bp)
Maize *gamete sterility*	S10M16C	**GAC TGC GTA CCA ATT CAG G**GA CAA CTT CCG TCG CTA CCA TGT GCC CGA GGG ACT GAT GAT AGT GAG GAA GGA GGA GTT TCT C**GT GTT ACT CAG GAC TCA TC**A A	103
	S10M9A	**GAC TGC GTA CCA ATT CAC A**TA ATT GCT AAT CAA TAC AAG GTG CCT TTC ATG GAT ATT **CAG TTA CTC AGG ACT CAT C**AA	78
	S12M1A	**GAC TGC GTA CCA ATT CAA C**AC GAC GAG GAA ACT AAT TTT CAC TTC CAT ATC AAC CAC TAA CAT TGA CAT TTC TTA TTT **GAG TTA CTC AGG ACT CAT C**AA	99
Wheat *eyespot resistance*	SK311	**GAT GAG TCC TGA GTA ACT G**AT AGG GCC AAT CAA TTA GAA AAG CAG AAT TAG GGG ATC ATC CAT CCA AGC AAG TAG TAA CTT GAC A**CC TGA ATT GGT ACG CAG TC**A A	106
Wheat *cytoplasmic male sterility*	SK35	**GAC TGC GTA CCA ATT CAG CCC** AAG TAG TAA TTT GGC CGA CCG GGC GCA TAG CTT CGA AGG TTT CCC ACT AGA GGC TAG CAG GGC CTT CGA GGT GAT AGG CAG CAT AAG TAA CCT TGT CGG CTT CGG CTA CGT TAG CAG AAC GTA GCT TGT GGG TAA TGC TGC GAA CCA ATC ATC CGC GTC GAG GGG CTC GGC GGA ATG GTT AAT TTA GTT TGC TAC TGA TTA CAT AAT GCC ACA GC**C TGT TAC TCA GGA CTC ATC** AA	257
	SK35AMS1	TGT TCC AAA CTG GAA CAA CAC TCA ACC CTA TCT CGG TCT ATT CTT TTG ATT TAT AAG GGA TTT TGC CGA TTT CGG CCT ATT GGT TAA AAA ATG AGC TGA TTT AAC AAA AAT TTA ACG CGA ATT TTA ACA AAA TAT TAA CGC TTA CAA TTT AGG TGG CAC TTT TCG GGG AAA TGT GCG CGG AAC CCC TAT TTG TTT ATT TTT CTA AAT ACA TTC AAA TAT GTA TCC GCT CAT GAG ACA ATA ACC CTG ATA AAT GCT TCA ATA ATA TTG AAA AGG AAG AGT ATG AGT ATT CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG GCA TTT TGC CTT CCT GTT TTT GCT CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT GCA CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA TGT GGC GCG GTA TTA TCC CGT ATT GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA ACA TGA GTG AAA AAA CTG GGG CCA ATT TAT TTC TGA CAA CGA TCG GGG GAC CGA AGG GCT AAC CGC TTT TTT GCC CAA CAT GGG GGG TAT GTA ACC CCC CTT GAT CGT TGG AAC CGA A	785
	SK36	ACT ACC AGT CAA ATG GCA TCT ACA GGA CGG CCA AGG TTT TGA AAT TTG CAT CTT TTG AAT CTA CAT ATT CTC TCT GCA TAT ATA TAT ATA CAT ATT GTT GTC CAT TCT GAA CTT AAC CCT GGA AGC ATC TGC TTT TGC TAG GTG GCC TTC TGC ATC CAC AAC ATC TCG TAC CAG GGC CGC TTC TCC TTC GAA	200

Selective amplification primer sequences in bold – ***Eco*RI** in red, ***Mse*I** in blue.

4.3.2 Optimisation of SCAR primer design

The validation results of the two primer pair designs used to amplify SCAR SK35 were used to design the primer pairs to amplify the other SCARs. Both primers pair design types were tested on wheat *cytoplasmic male sterile* and *restorer* genotypes C4, C5, C6 and CR41.

The two primer pair designs produced different results. The primer set that excluded the restriction enzyme sequences produced a number of amplification products, but none of them were of the expected size. The primer set that included the restriction enzyme sequence, however, produced the expected amplification fragment from the DNA of genotype CR41. This is an indication that a primer containing the restriction enzyme sequence anneals more accurately than one without the restriction enzyme sequence (Figure 4.1) (Table 4.2).



A B

Figure 4.1 Amplification products of SCAR SK35 primers that excluded (A) and included (B) the restriction enzyme sequence from wheat *cytoplasmic male sterility* genotypes.

The expected size of the amplified SCAR is determined by calculating by the number of nucleotides from the beginning of the forward primer binding site sequence to the end of the complementary binding site sequence of the reverse primer. The primer pair that included the restriction enzyme sequence would therefore produce an amplification product that is longer than the primer pair that

excludes it. The sizes of the different amplification products are presented in table 4.1.

Table 4.2 Amplification products of SCAR SK35 using the two primer pair types.

Primer type	Primer sequence*	Size of expected amplified SCAR (bp)	Genotypes tested	Size of observed amplified SCAR (bp)
Restriction enzyme sequence excluded	F: 5'-CAG CCC AAG TAG TAA TTT GG-3' R: 5'-ACA GGC TGT GGC ATT ATG TA-3'	233	C4, C5, C6 and CR41	1 000
			C4, C5, C6 and CR41	700
			C4, C5, C6 and CR41	550
Restriction enzyme sequence, *Eco*RI and *Mse*I included.	F: 5'-GAA TTC AGC CAA AGT AGT AA-3' R: 5'-TTA ACA GGC TGT GGC TTA TA-3'	225	C4, C5, C6 and CR41	550
			CR41	233

* *Eco*RI restriction sequence indicated in red.

* *Mse*I restriction sequence indicated in blue.

These results (Table 4.2) indicated that the primer pair that included the restriction enzyme sequence produced more accurate SCAR amplification products than the primer that excluded the restriction enzyme sequence. Therefore, for all subsequent SCAR testing, primer pairs that contained the restriction enzyme sequences were used (Table 4.3).

Table 4.3 Primer pairs designed to amplify SCARs.

Trait	Primer pair		Primer sequence*	Size of expected amplified SCAR (bp)	Tm
Maize *gamete sterility*	P10M16C	F:	5'-GAA TTC AGG GAC AAC TTC-3'	78	56°C
		R:	5'-TTA ACA CGA GAA ACT CCT CC-3'		58°C
	P10M9A	F:	5'-GAA TTC ACA TAA TTG C-3'	53	42°C
		R:	5'-TTA ACT GAA TAT CCA TG-3'		42°C
	P12M1A	F:	5'-GAA TTC AAC ACG ACG AGG-3'	81	54°C
		R:	5'-TTA ACT CAA ATA AGA AAT GAC-3'		52°C
Wheat *eye spot resistance*	PK311	F:	5'-GAA TTC AGG TGT CAA GTT AC-3'	81	56°C
		R:	5'-TTA ACT GAT AGG GCC AAT CA-3'		56°C
Wheat *cytoplasmic male sterility*	PK35	F:	5'-GAA TTC AGC CAA AGT AGT AA-3'	233	56°C
		R:	5'-TTA ACA GGC TGT GGC TTA TA-3'		56°C
	PK35AMS1	F:	5'-CAA CAC TCA ACC CTA TCT-3'	745	56°C
		R:	5'-CAA GGG GGG TTA CAT ACC-3'		60°C
	PK36	F:	5'-GCA TCT ACA GGA CGG CCA-3'	162	58°C
		R:	5'-GCC CTG GTA CGA GAT GTT-3'		56°C

F: Forward primer

R: Reverse primer

* *Eco*RI restriction sequence indicated in red.

* *Mse*I restriction sequence indicated in blue.

4.3.3 Validation of SCAR primers

Maize

Two of the three amplified SCAR fragments were the expected size in the genotype that contained *gamete sterility* GAS 12 (+). The amplification product produced from the genotype without *gamete sterility* was either larger or smaller than the expected. It was therefore easy to distinguish between the two genotypes. These results were substantiated in the related *gamete sterility genotype* GAS 10 (+). The amplification products produced by the SCAR primers of S10M16C were of the same size for all the genotypes and it was thus not able to distinguish between them (Table 4.4).

Table 4.4 Amplification products of the maize SCAR primer pairs.

SCAR	Genotypes	Size of observed amplified SCAR (bp)	Potential to distinguish between genotypes
S10M9A	GAS 12 (+)	**53**	Yes
	GAS 10 (+)	**53**	
	GAS 9 (-)	>53	
S12M1A	GAS 12 (+)	**81**	Yes
	GAS 10 (+)	**81**	
	GAS 9 (-)	<81	
S10M16C	GAS 12 (+)	**78**	No
	GAS 10 (+)	**78**	
	GAS 9 (-)	**78**	

Expected sizes in bold.

> Fragment larger than expected.

< Fragment smaller than expected.

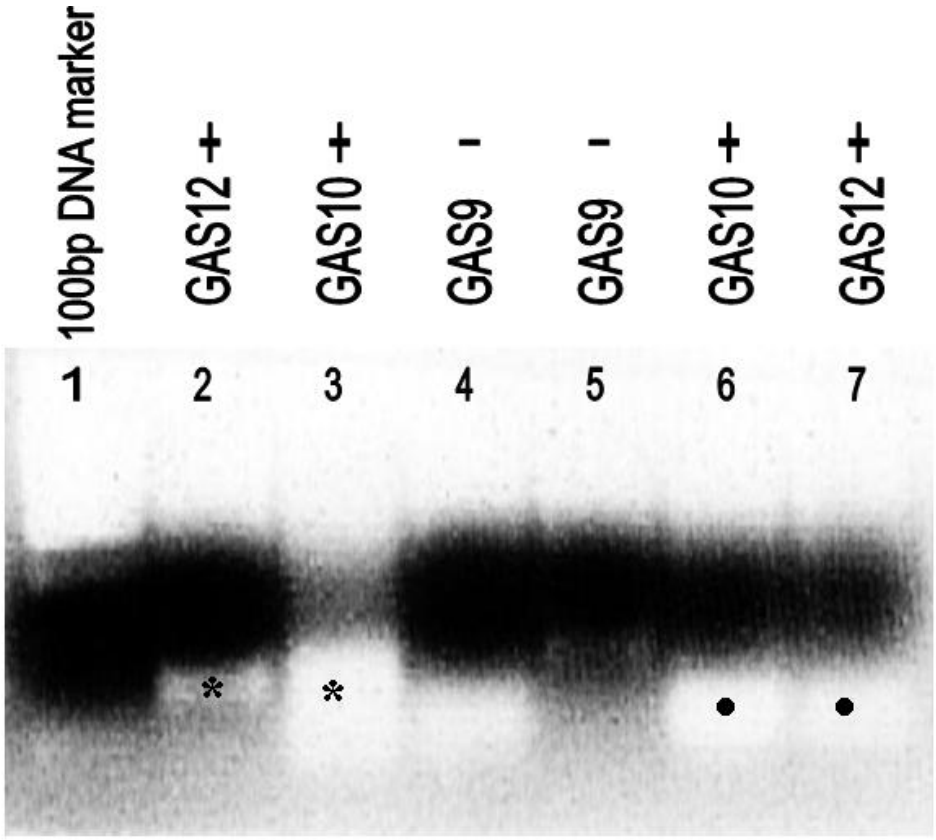


Figure 4.2 Amplification of the SCAR that identifies maize *gamete sterility*. Lanes 2-4 is the SCAR S12M1A and lanes 5-7 S10M9A.

***** SCAR S12M1A observed amplified fragment of 81 bp

● SCAR S10M9A observed amplified fragment of 53 bp

Wheat

Primer pair K311 that was designed to amplify SCAR SK311, marker for wheat *eye spot resistance*, successfully distinguished between *eye spot resistant* genotypes (E9 and E13) and *eye spot susceptible* genotypes (E10 and E12). The primer pair amplified a single fragment of the expected size of 81 bp in both the two resistant genotypes, while no amplification occurred in the susceptible genotypes (Figure 4.3).

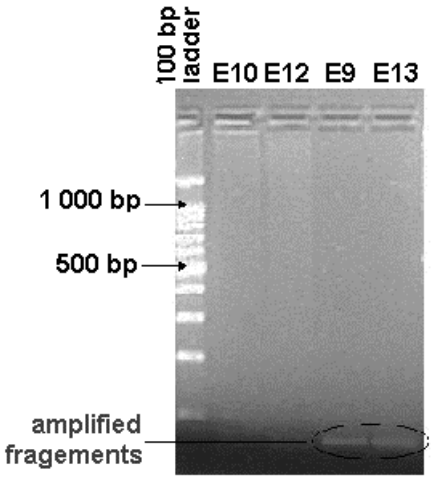


Figure 4.3 Amplification of the wheat *eye spot resistance* SCAR SK311 with primer set PK311.

Primer pair PK36 that was designed to amplify SCAR SK36, marker for wheat *cytoplasmic male sterility*, successfully distinguished between the *restorer* genotypes (C6 and CR41) and the *male sterile* genotypes (C4 and C5). However this primer pair not only amplified the expected size of 162 bp in both the two *restorer* genotypes, it also amplified two other fragments in all the genotypes. (Figure 4.4).

The two primers pairs PK35 and PK35AMS1 that were designed to amplify SCARs SK36 and SK35AMS1 respectively were able to amplify fragments in only one of the two *restorer* genotypes, CR41, but not in the other, C6. The primer pair PK35 amplified the expected fragment of 233 bp together with one other anonymous fragment in all the genotypes. Primer pair PK35AMS1, on the other hand, amplified the expected fragment of 745 bp, but in this case a variable number of anonymous fragments were also discerned for the different genotypes (Figure 4.4).

Four out of the seven designed primer pairs were able to amplify a SCAR of the correct size (P10M9A, P12M1A, PK311, and PK36). Two were able to amplify a SCAR in at least one of SCAR containing genotypes (PK35 and PK35AMS1), and one could not discriminate between the genotypes (P10M16C). A summary of the performances of the different primer pairs are presented in table 4.5.

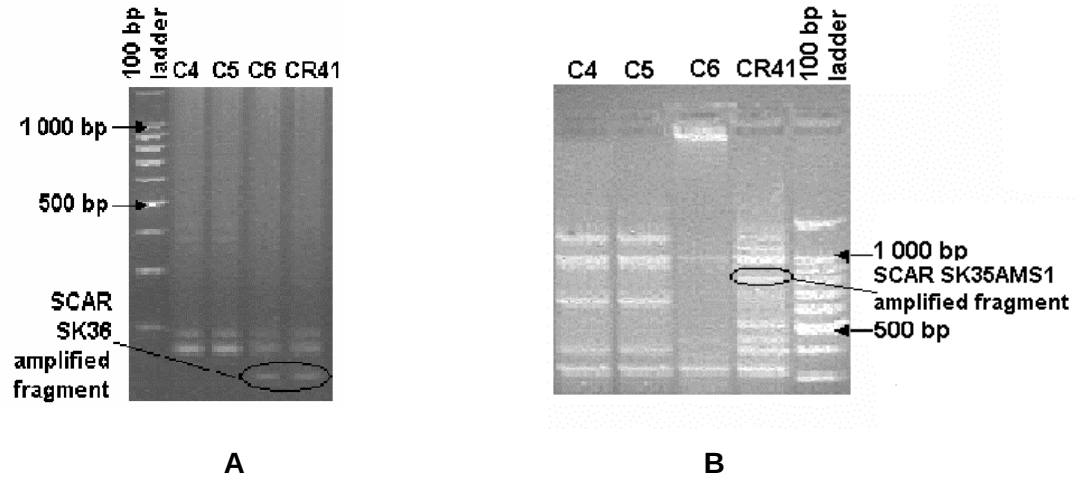


Figure 4.4 Amplification of the wheat *cytoplasmic male sterility* SCARs SK36 (A) and SK35AMS1 (B) with primer pairs PK36 and PK35AMS1 respectively.

Table 4.5 SCAR primer pair performance.

Primer pair	Genotypes showing SCAR amplification	Size of amplified SCAR (bp)	SCARs suitable to identify genotypes
P10M16C	GAS 9 and GAS 12	78	No
P10M9A	GAS 12	53	Yes
P12M1A	GAS 12	81	Yes
PK311	E9 and E13	81	Yes
PK35	CR41	233	No
PK35AMS1	CR41	745	No
PK36	C6 and CR41	162	Yes

4.4 DISCUSSION

The development of the SCAR technology has enhanced the power of MAS (marker assisted selection) significantly. The isolation of a particular fragment in a fingerprint, then using it to probe the presence of the genetic information of a particular trait in a genotype bypasses laborious analyses of fingerprints (Sari-Gorla, *et al.*, 1996). This technology provides for direct amplification of a marker and an immediate answer to the question whether the SCAR marker is present or not.

In both maize and wheat, this technology has been used in breeding programmes with great success in. In maize, a RFLP probe linked to the recessive gene of resistance to *southern corn leaf blight* was converted to a SCAR and used to introduce new inbred lines in a breeding programme (Zaitlin *et al*., 1993). In the same way, an AFLP fragment linked to the *phb1* deletion in wheat was converted to a SCAR and used for scoring of this deletion in wheat cultivars (Qu, *et al*., 1998).

One of the most important aspects in the development of a SCAR probe is the design of suitable primers. This investigation clearly shows that the correct primer sequence produces superior results. Furthermore, primers should have a significant number of nucleotides, 15 bp - 21 bp, so that together with a high annealing temperature the reaction is highly specific.

Once suitable primers have been identified, their probing ability should be validated. This requires extensive testing; firstly on the genotypes from where they originated and thereafter in a wide range of genotypes to establish reliability. Extensive testing was beyond the scope of this investigation; however the results clearly support the importance of this step, as not all the primer pairs could be used to exclusively identify trait containing genotypes.

In wheat that consists of three homoeologous genomes primer testing should be more rigorous than for maize. The danger exists that very closely related loci exist

in more than one of the genomes, and not only in the genome from where it was originally isolated. This complication of the polygenomic nature of wheat was demonstrated by the multi-allelic amplification of some SCARs. Only one of the wheat primer pairs (PK311 for wheat *eyespot resistance*) amplified the single expected fragment in the trait containing genotypes. Another primer pair (PK36 for wheat *cytoplasmic male sterility*) was also able to distinguish between the genotypes that contained the trait and those that did not, but also amplified two additional anonymous fragments. A similar result was obtained for PK35 (wheat *cytoplasmic male sterility*), accept that the fragment of interest was only amplified in one of the trait containing genotypes and not in the other. The most variable amplification results were obtained from primer pair PK35AMS1 (wheat *cytoplasmic male sterility*) where the fragment of interest was amplified in one of the genotypes that contained the trait, but not in the other. In this case, however, a number of anonymous fragments were amplified in all the genotypes in varying numbers.

It is clear from these results that the multi-genomic nature of wheat is a complicating factor in the development of SCAR technology and requires extensive testing before put into practice.

used in this investigation. There may have been one (or more) parent(s) in common.

Another interesting outcome of this investigation was the great size similarity of fragments related to a particular trait, which probably indicates that some primer combinations anneal rather close to one another, producing fragments of the same, or nearly the same size.

Within each of the groups of polymorphic fragments, it was also possible to identify putative markers linked to the traits of interest (in coupling). However, the investigation did show that AFLP fragments may be polygenic in nature, which indicated the need to evaluate fragments isolated from fingerprints before any cloning and testing is undertaken. Eventually extensive testing will be required in order to ascertain whether the genetic linkage is small enough to be useful.

In this study, it was shown that for both maize *gamete sterility* and wheat *eye spot resistance*, a low level of polymorphism was observed. The low level of polymorphism may be an indication of the genetic similarity of the near isogenic lines that was used. This observation is shared by a similar study in wheat by Najimi, 2002. In wheat *cytoplasmic male sterility* no amplification loci could be identified that were in coupling. This is an indication that here might be no cytoplasmic DNA in the total DNA extraction done, and that the differences that were observed, were between the nucleic DNA of the samples. Since the samples for wheat *cytoplasmic male sterility* is even more genetically related than NILs used in the other two traits, it is understandable that the level of polymorphism is even lower (Mueller and Wolfenbarger, 1999).

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Plant breeding, the art of improving the genetic make-up of plants for the benefit of humankind, has been practised for many centuries. Today, plant breeding is a complex and sophisticated industry, utilising a variety of technologies that were not available to the plant breeder just a few decades ago. These modern technologies, such as genome analysis (genomics), mutation induction, marker-assisted selection (MAS), tissue culture, gene transfer and various other biotechnologies have greatly facilitated the rapid growth of the industry.

Crop improvement has involved selective breeding and hybridisation using controlled pollination of plants. The development of biotechnology tools changed traditional plant breeding into a far more precise science. Traditional plant breeding involved the crossing of hundreds or thousands of genes, plant biotechnology now allows for the transfer of only one or a few desirable genes. However, there are difficulties in introducing new traits into plants: the number of generations needed (many years) for introduction of a trait; many traits are quantitatively inherited (controlled by several genes); traits may be difficult or expensive to see directly (for example resistance); and many of the traits are sensitive to genotype-environment interactions. Amongst the initiatives to overcome this, a range of biotechnological methods have found their way into plant breeding through the last two decades. These include DNA marker systems and associated techniques.

Some advantages of DNA markers are that they are not influenced by the environment, are expressed in all tissues and can be detected at all stages of plant growth. A large number of markers have been developed, and a number of these are reviewed in Gupta *et al*. (1999) and Hartl (2000). Amplified fragment length polymorphisms (AFLPs) involve the selective amplification of restriction fragments from a digest of total genomic DNA using PCR (Vos *et al*. 1995). Although the degree of polymorphism is high for AFLPs, there have been problems to detect adequate

and useful polymorphisms in plants. This means that a lack of polymorphism in especially breeding populations can be an obstacle for using DNA markers. The AFLP technique is expensive, technically difficult, laborious and time consuming to set up, however, in return it is reliable, reproducible and highly informative (Powell *et al*., 1996; McGregor *et al*., 2000). The marker system of choice depends on the objective of the study plus skills and facilities available in the laboratory (Milbourne *et al*., 1997).

The applications of DNA markers are numerous; they can be used for gene tagging and genome mapping. Mapping is putting markers in order, indicating the relative genetic distances between them, and assigning them to their linkage groups on the basis of the recombination values from all their pair-wise combinations (Jones *et al*., 1997). By the use of marker techniques it is possible to localise qualitatively as well as quantitatively inherited traits. These quantitative traits are also referred to as quantitative trait locus/loci (QTL). Many important traits are quantitatively inherited like for instance yield. The environment often influences quantitative traits. Furthermore improvement of crop species for quantitative traits is difficult because the effects of individual genes controlling the traits cannot be readily identified. Identification of the individual genes could be very useful. Among other things it could improve the efficiency of selective breeding especially for traits with low heritability.

Marker-assisted selection (MAS) can assist in selecting suitable parents by examination of variation in genetic origin and presence of desired traits in different lines. It is important to choose breeding lines which contain desired traits and at the same time are diverse in origin to make sure that as many different alleles as possible are combined in breeding programmes. MAS is a breeding strategy that applies indirect selection. Instead of selecting for the gene itself the molecular markers closely linked to the genes of interest are used to monitor the incorporation of the desirable alleles from the donor source (Dudley, 1993). Marker screening within the early generations of a breeding programme means that MAS can help to speed up backcrossing and develop improved lines or populations. It may especially have advantages in some cases where phenotypic selection is difficult. For instance, marker screening of DNA of seedlings can be a less time-, space- and resource

demanding process than carrying out full-scale disease sensitivity trials at each stage. Moreover sometimes it is impossible or inconvenient to use the disease or pest for direct screening. MAS can also be used for selecting several traits simultaneously, for example with pyramiding of different resistance genes in one elite line (Liu *et al*., 2000).

In this investigation the AFLP technology was utilised with maize and wheat commercial cultivars. The aim was to identify putative markers linked to *gamete sterility* in maize and *eye spot resistance* and *cytoplasmic male sterility* in wheat, and to develop primers that could be used as a diagnostic tool in the identification of the markers in different genotypes that are linked to the different traits.

The complexity of fingerprints of maize and wheat differed greatly due to the wheat genome being far more complex than that of maize. Wheat, an allopolyploid, consists of three different genomes each having originated from a different ancient parent. The high AT content which is generally found in many plant genomes necessitated the careful selection of primer extensions (Thomas *et al*., 1995; Cervera *et al*., 1996a). Increasing the GC content of the primers could result in fewer fragments (Keim *et al.*, 1997; Qi and Lindhout, 1997). In this study, maize *gamete sterility* showed a downward trend in the amount of amplified loci with an increase in GC content of primers, whereas for wheat *eye spot resistance* showed an increase in the amount of amplified loci with an increase in GC content of primers.

The AFLP fragments generated were within the expected size range of between 50 bp and 900 bp (Vos *et al.,* 1995), providing sufficient usable fragments in the size range of 50 to 200 bp. It was interesting to note that the different complexities of the genomes influenced the outcomes of the various fingerprints. Fingerprints of wheat *eyespot resistance* displayed 99 bands, which were expected for a crop with such a complex genomic constitution, to only 42 for wheat *cytoplasmic male sterility,* indicating the close relatedness of the genotypes carrying this trait. Maize fingerprints, on the other hand, produced an intermediate number of fragments of 60 which was expected for a genome of the size of maize (Cho *et al.*, 1996; Nakajima *et al.,* 1998).

The number of polymorphic loci generated by the different primer combinations varied greatly. 72,7 % of the primer combinations for maize *gamete sterility* produced polymorphic loci, while in wheat the primer combinations produced substantially less. Polymorphic loci was produced for 56,2 % of the primer combinations used in *eyespot resistance* and for *cytoplasmic male sterility* 17,2 % of the primer combinations produced polymorphic loci. These results revealed that the near isogenic lines of wheat were genetically different enough in the case of *eyespot resistance,* but not the case for cytoplasmic *male sterility.* When primers with a 50 % GC content was scored, the number of polymorphic loci in maize increased indicating the polymorphic nature of the AT rich regions of the genome (Röder *et al.,* 1995). This was not the case for wheat which could mean a low polymorphic content, but is probably due to the more complex nature of the genetic constitution of wheat and may require a more in depth analysis of techniques.

Polymorphic markers linked to the maize and wheat traits were successfully generated and evaluated. The number of polymorphic fragments was far greater in maize than in wheat; 81 % for maize *gamete sterility* and 67 % for wheat *eye spot resistance,* also supporting the *primary* difference in the genomic constitution of these two corps. Maize, a diploid with 10 chromosome pairs (x = 10) and wheat, a hexaploid, that consists of three different genomes (AABBDD, 2n = 42) (Poehlman, 1987). Furthermore, these results also suggest extensive similarity (homoeolology) between the wheat genomes (A, B and D) and the presence of homologous loci over the three genomes (Van Deynze, 1995).

Putative markers linked to the traits of interest were identified for all three traits. For *maize gamete sterility*, seventeen putative markers were identified, thirty two for *wheat eyespot resistance*, and six for *wheat cytoplasmic male sterility*.

The development of the SCAR technology has enhanced the power of MAS (marker assisted selection) significantly. The isolation of a particular fragment in a fingerprint, then using it to probe the presence of the genetic information of a particular trait in a genotype bypasses laborious analyses of fingerprints (Sari-Gorla, *et al.*, 1996). This

technology provides for direct amplification of a marker and an immediate answer to the question whether the SCAR marker is present or not. Putative markers were selected for each of the three traits, cloned, characterised and primers designed. Primer pairs of a length of 15 bp-21 bp that included the restriction site produced the best results. Although extensive testing of the primers is beyond the scope of this investigation, preliminary results have shown that the polygenomic nature of wheat causes multi-allelic amplification of some SCARs. This suggests that primer testing in wheat be more rigorous due to the close genetic relationship of the genomes. The most reliable results in this preliminary investigations showed that only one of the wheat primer pairs for wheat *eyespot resistance* amplified the single expected fragment in the trait containing genotypes. One primer pair could, however, distinguish between the genotypes that contained wheat *cytoplasmic male sterility* and those that did not, but also amplified two additional unidentified fragments.

Markers are predicted to find widespread application for genome tagging, linkage mapping, MAS, QTL analysis and many more. The actual benefit in developing plants with new traits using these techniques is continually growing as the techniques become more widely used in general plant breeding (Moreau *et al.*, 2000). Marker systems need refinement to make them more user friendly, effective and economical viable while still keeping high reproducibility across laboratories. However, progress will be speeded up as automated DNA-sequencers become more available.

Despite problems and difficulties it seems convincing that PCR based DNA markers and associated techniques can supplement conventional methods to make future plant breeding more effective. Markers can especially find application in areas where conventional methods are not sufficient, like utilisation of quantitatively inherited traits and agronomically interesting genes from wild species. Once linkage maps have been created, MAS will be able to help define up the process of breeding. Together, marker techniques and conventional methods may accelerate genetic modification, stabilise yield and increase total food production to supply the needs of the growing human population.

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APPENDIX A

BUFFERS AND SOLUTIONS

Standard buffers and solutions were prepared as outlined in Sambrook *et al.* (1989). Solutions were autoclaved at 121°C for 15 min or were filter sterilised through a 0.22 µm Millipore filter.

1 X TE (TRIS-EDTA) BUFFER (pH 8,0)

Tris base	1,21 g
EDTA (0,5 M; pH 8,0)	2,0 ml
Distilled water to	1,0 L

The pH was adjusted to 8,0 with 0,1 M HCl

50 X TAE (TRIS ACETATE EDTA) BUFFER (pH 8,0)

Tris base	242,0 g
Glacial acetic acid	57,1 ml
EDTA (0,5 M; pH8,0)	100,0 ml
Distilled water to	1 L

1 X TAE (TRIS ACETATE) BUFFER (pH 8,0)

Add 20 ml 50 x TAE buffer to 750 ml distilled water. Adjust pH to 8,0 with 0,1 M HCl. Make to final volume of 1 L with distilled water.

10 X TBE (TRIS-BORATE EDTA) BUFFER (pH 8,0)

Tris base	108,0 g
Boric acid	55,0 g
EDTA	20,0 ml
Distilled water to	1 L

1 X TBE (TRIS-BORATE EDTA) BUFFER (pH 8,0)

Add 100 ml 10 x TBE buffer to 750 ml distilled water. Adjust pH to 8,0 with 0,1 M Boric acid. Make to final volume of 1 L with distilled water.

GEL ELECTROPHORESIS LOADING BUFFER

Bromophenol blue	62,5 g
Sucrose	10,0 g
EDTA (0,5 mM; pH 8,0)	1,0 ml
Distilled water to	25,0 ml

ALKALINE LYSIS BUFFERS FOR PREPARATION OF PLASMID DNA

Solution I

50 mM glucose

25 mM Tris-Cl (pH 8,0)

10 mM EDTA (pH 8,0)

Solution II

0.2 N NaOH (freshly diluted form a 10 N stock)

1 % SDS

Solution III

5 M potassium acetate	60,0 ml
Glacial acetic acid	11,5 ml
Distilled water	28,5 ml

RESTRICTION ENZYME BUFFERS

Boehringer Mannheim buffers B and M were used with the appropriate restriction endonuclease. Final concentration in mmol/l

STOCK SOLUTION	B	M
Tris-HCl	10	10
$MgCl_2$	5	10
NaCl	100	50
Dithioerythritol	-	1
2-mercaptoethanol	1	-
pH at 37°C	8,0	7,5

6% PAGE MIX

A 6 % polyacrylamide gel mix was used.

Urea	12,0 g
Acrylamide	4,8 g
Bis-acrylamide	0,21 g
10 X TBE	8,0 ml
Distilled water to	80,0 ml

55 ml of the mix was filtered through a 0,8 µm Millipore filter. TEMED (50 µl) and 50 µl of 50 % ammonium persulphate was added and mixed before the gel was poured.

MEDIA

Solid media contained 1,5 % (w/v) agar. Media were autoclaved at 121°C for 20 min.

Luria-Bertani medium (LB)

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water to	1 L

ANTIBIOTICS AND MEDIA ADDITIVES

	Concentration	Stock
Ampicillin	100 µl/ml	100 mg/ml in water

Stock solutions of antibiotics dissolved in water were sterilised by filtration through a 0,22-micron filter. All antibiotics were divided into 1 ml aliquots and stored at -20°C.

IPTG (ISOPROPYL-B-D-THIO-GALACTOPYRANOSIDE)

IPTG	23,4 mg
Distilled water	1,0 ml

The soluton was aliquoted and stored at -70°C.

X-GAL (5-BROMO-4-CHLORO-3-INDOLYL-B-GALACTOCIDE)

X-gal	0.2 g
Dimethylformamide	10 ml

The solution was stored at -70°C.

MAIZE DNA EXTRACTION BUFFER

100 mM Tris-Cl pH8,0	100ml of 1M stock
50 mM EDTA	100ml of 0,5M stock
500 mM NaCl	100ml of 5M stock
10m M β-ME	Add just before staining: 1 µl/ml

CTAB BUFFER

5 % ($^{w}/v$) CTAB	50,0g
1,4 M NaCl	81,8g
0,2 % ($^{v}/_{v}$) 2-mercapto-ethanol	2ml
20 mM EDTA	7,44g
100 mM Tris-HCl (pH8,0)	100ml of 1M Tris.HCl (pH 8.0) stock
1 % PVP	10g

ELUTION BUFFER FOR DNA FRAGMENTS FROM PAGE GELS

0.5 M Ammonium acetate 96.35g

10 mM Mg acetate	26.8g
1 mM EDTA (pH8,0)	46,5g
0.1 % SDS	25g

Dissolve to a final volume of 1l deionised water.

CHLOROFORM : ISOAMYL ALCOHOL (24:1) 1l

Chloroform	960ml	480ml
Isoamylalcohol	40ml	20ml