PT520
Molecular markers for PCN resistance

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Know-how for Horticulture™

PT520

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1.0 Introduction

Molecular markers have the potential for increasing the efficiency of breeding programmes by allowing for early selection of important traits.

This is the final report for APIC\HRDC project **PT520** *'Molecular markers for PCN resistance'*, a collaborative project between Agriculture Victoria (Institute for Horticultural Development-Knoxfield) and CSIRO (Division of Plant Industry).

The authors thank APIC and HRDC for funding this research and Graeme Wilson (IHD-Toolangi) for providing most of the plant material used.

This report is divided into four sections;

- Industry and technical summaries
- Evaluation of a diagnostic system, based on the Polymerase Chain Reaction (PCR) that has the potential to be diagnostic for screening potato plants for their response to Potato Cyst Nematode (PCN *Globoderra rostochiensis*)
- Evaluation of alternative approaches using DNA hybridisation and Random Amplified Polymorphic DNA (RAPDs)
- Recommendations

Attached are four appendices, including an introduction to molecular markers and PCR and a glossary of terms.

1.1 Industry summary

Potatoes are a major horticultural crop which would benefit if biotechnological approaches could be suitably used.

The discovery of Potato Cyst Nematode (PCN *Globodera rostochiensis*) in Western Australia in 1986 and Victoria in 1991 has, in part, resulted in breeding for resistance to PCN becoming an objective of the National Potato Improvement and Evaluation Scheme (NaPIES).

Breeding for resistance to PCN is time consuming and expensive, as material has to be sent abroad for screening because PCN is a quarantinable disease in Australia.

Therefore methods that result in selection for PCN resistance at the seedling stage will benefit NaPIES and ultimately the potato industry.

Recently a genetic map for potato has been produced by scientists in Europe and the USA. An important finding has been a marker that is linked to the *H1* gene, which is used as a source of resistance to PCN pathotypes Ro1 and Ro4.

This marker has been used to screen a range of potato cultivars and seedlings.

Initial research with the cultivars Atlantic and Maris Piper found that the marker system operated as predicted.

Additional work, screening a population of advanced breeders lines, which had been screened for their response to PCN with nematode challenge experiments found no consistent relationship between the nematode challenge and marker approach. This was extremely disappointing.

One of the expectations of this project was the capability to screen plants at the seedling stage so that susceptible lines could be discarded. Three hundred seedlings, resulting from a cross between Rosa (PCN resistant) and Snowgem (PCN susceptible) were screened, but no clear relationship could be predicted.

Two other approaches were evaluated, both of which have promise for early selection for PCN resistance.

1.2 Technical summary

The discovery of Potato Cyst Nematode (PCN *Globodera rostochiensis*) in Western Australia in 1986 and Victoria in 1991 has resulted in breeding for resistance to PCN becoming an objective of the National Potato Improvement and Evaluation Scheme (NaPIES).

Resistance breeding for PCN is time consuming and expensive as material has to be sent abroad for screening by nematode challenge. Therefore, methods that improve the efficiency of screening would benefit the potato industry. Restriction Fragment Length Polymorphism (RFLP) analysis has generated number of genetic maps for potato and a marker (CP113) found that cosegregates with the *H1* locus, which confers resistance to PCN pathotypes Ro1 and Ro4, has been mapped to chromosome V. The CP113 marker has been sequenced and primers designed and a Polymerase Chain Reaction (PCR) test evaluated.

The PCR test utilises three primer sets. Primer set 1 is able to detect the *a*, *b* or *d* alleles. Digesting the generated fragment with *Taq* 1 results in an *a* allele specific fragment. Primers sets 2 and 3 detect the *a* allele.

The PCR system was optimised using the cultivars Atlantic and Maris Piper and the predicted results obtained. Results with primer set three were inconsistent.

Using the PCR system with a number of advanced lines that had been screened for their response to PCN pathotype Ro1 or unscreened seedlings from a segregating population, no consistent results were obtained that could predict the response of germplasm to PCN. While disappointing, the unpredictability could be due to the presence of other genes associated with PCN resistance in the lines tested and recombination between *H1* and CP113.

Two other approaches were evaluated. Another marker (CD78) was also found to be closely linked to *H1*. Preliminary studies using DNA hybridisation could not detect a clear relationship with plants of known response to PCN. Further work is warranted as it has been shown to be useful with tetraploid germplasm. Methods based on Randomly Amplified Polymorphic DNA (RAPDs) have been evaluated and polymorphisms detected. Such an approach would be useful if bulk segregant analysis could be used.

This work has shown the molecular marker system based on CP113 cannot be readily applied to NaPIES for the routine screening for PCN. Preliminary evaluation of the CD78 marker found it potentially useful. Further work is warranted, but only if breeding for PCN resistance becomes a priority of NaPIES rather than an objective.

2.0 Introduction

Classical plant breeding is based on selection of superior individuals among the segregating progeny of a sexual cross. Selection is usually based on a visible phenotype (e.g. plant vigour or general appearance) or on measurable traits (e.g. yield and starch composition). Selection for characteristics such as pest and disease resistance is more difficult and achieved by challenging progeny with the organism of interest. Many of the complications of phenotype based selection can be mitigated by direct selection for genotype using molecular markers that co-segregate with the genes of interest (Burr 1994).

Since the detection of Potato Cyst Nematode (PCN *Globodera rostochiensis*) in Western Australia in 1986 and Victoria in 1991 it has had considerable social and economic impact on the Australian potato industry (Annon 1993) and breeding for resistance has become an objective of the National Potato Improvement and Evaluation Scheme (NaPIES).

The *H1* gene confers resistance to PCN pathotypes Ro1 and Ro4 (Kort *et al.* 1977) which has been introduced into potato cultivars as a single dominant gene from *Solanum tuberosum* ssp. *andigena* and mapped to chromosome V (Gebhardt *et al.* 1993). Other resistance genes include *Gro1* from the diploid species *S. spegazzinii* which has been mapped to chromosome VII (Barone *et al.* 1990) and confers resistance to pathotype Ro1 and the *Fa* and *Fb* (*Gro1*) genes (Ross 1986). The *H1* gene has been incorporated into a number of widely grown cultivars, including Atlantic and Maris Piper. In the USA the *H1* gene is the main, possibly only, source of resistance used and in excess of 20 cultivars released in the last 20 years carry this gene (Pineda 1993).

Plant breeding is a numbers game and it is often necessary to screen many plants to find those with the most desirable combination of characteristics. Therefore approaches that result in the selection of plants with desirable features, as early as possible, should be attractive to breeders. For many years breeders have used a number of approaches to improve the efficiency of breeding programmes. One of the most widely used is linkage analysis, which uses a marker that segregates with a character of interest. Markers are usually for a morphological character such as leaf colour or a chemical such as an enzyme. Once such a marker has been associated or linked with a characteristic, it can be used to monitor for the presence of the character unless the linkage has been lost. Such endeavours have proved to be extremely useful as it has resulted in the development of genetic maps where characteristics have been assigned to particular chromosomes. More recently the development of genetic maps has been hastened by using restriction fragment length polymorphism (RFLP) analysis (Beckmann and Soller 1985). RFLP analysis has been used to develop a number of genetic maps of potato (Bonirabale *et al.* 1988; Gebhardt *et al.* 1989; Jacobs *et al.* 1995). These maps have been used to identify markers that are linked to important agronomic traits that confer resistance to Potato Virus X (Ritter *et al.* 1991), *Phytophthora infestans* (Leonards-Schippers *et al.* 1992) and PCN (Barone *et al.* 1990; Gebhardt *et al.* 1993; Kreike *et al.*1993).

The *H1* locus is closely linked to RFLP locus CP113 (Gebhardt *et al.* 1993). When heterozygous dihaploid potato lines are crossed, CP113 segregates as four possible alleles; *a*, *b*, *c* and *d*. Allele *a* segregates in coupling with *H1* allele, whilst *b*, *c* and *d* are linked to susceptibility alleles (Niewöhner *et al.* 1995).

2.1 Background

A PCR test that is 'diagnostic' for the *a* allele has been developed that involves three different primers sets (Niewöhner *et al.* 1995). Primer set one (CP113-5' 1\CP113-3' 1) amplifies DNA within the marker locus CP113. The size of the amplified fragment varies, depending on the alleles present. Alleles *a*, *b* and *d* correspond to a single fragment of 1020 base pairs (bp), whereas allele *c* is detected as a pair of fragments of 530 and 480 bp. In addition, allele *a* can be distinguished from alleles *b*, *c* and *d* if the amplified fragment is digested with the restriction enzyme *Taq* 1. This digest produces an allele *a* specific restriction fragment of 340 bp.

Primer set two (CP113-5' 2\CP113- 3' 2) and three (CP113-5' 1\CP113-3' 3) only amplify DNA associated with allele *a*. With primer set two, a fragment of 760 bp is produced, whereas with primer set three, the fragment is 700 bp.

2.3 Experimental approach

The approach used for this project was to evaluate the three primer sets, initially with the cultivars Maris Piper and Atlantic. During this developmental phase a number of factors that could influence the result were evaluated (see below). The procedures were then used to screen different populations of potato.

The sequence of the primers used, there annealing temperatures and predicted response are detailed in Appendix 1.

When developing any diagnostic procedure it is important to test variable that could affect the reliability of the system. Factors known to influence PCR procedures include the quality of the DNA used and the components of the reaction. Appendix 2 assesses a number of DNA isolation procedures with the objective of having a method that is reliable, reproducible and allows many samples to be processed simultaneously. Appendix 3 details experiments aimed at developing favourable conditions for the three primer sets with Maris Piper and Atlantic. These conditions, often referred to as 'optimal', evaluated such factors as Mg concentration, dNTP concentration, polymerase type and template concentration.

3.0 System development with Maris Piper and Atlantic

3.1 Introduction

The PCR assay developed by Niewöhner *et al.* (1995) was evaluated using Maris Piper and Atlantic. Details of the developmental aspects can be found in Appendix 3 and the presented data summarises the essential parts.

3.2 Primer set CP113-5' 1\CP113-3' 1

From the series of experiments detailed in Appendix 3, the conditions in Table 1 were considered suitable for amplification of potato DNA with primer set CP113-5' 1\CP113-3'1

Component	Concentration
DNA	100 ng
$MgCl_2$	3 mM
Primer	0.25 µM
dNTPs	100 µM
Ampli*Taq* (Perkin Elmer)	1 U
Buffer	10 mM Tris, 50 mM KCl (pH 8.3)

Table 1. Suitable conditions for amplification with primer set CP113-5' 1\CP113-3'1

A typical result using Maris Piper and Atlantic DNA is shown in Figure 1, with the CP113 locus specific fragment of 1020 bp arrowed.

Figure 1. PCR products amplified from Maris Piper and Atlantic DNA using primer set CP113-5' 1\CP113-3' 1 and the optimised conditions

Lane 1 = Maris Piper
Lane 2 = Atlantic
Lane 3 = blank
Lane 4 = 100 bp marker

3.3 Restriction digest with *Taq* 1

The expected 340 bp restriction fragment (arrowed) was produced when the 1020 bp fragment generated from Atlantic DNA was digested with *Taq* 1 (Fig. 2). The same result was obtained with Maris Piper DNA (not presented).

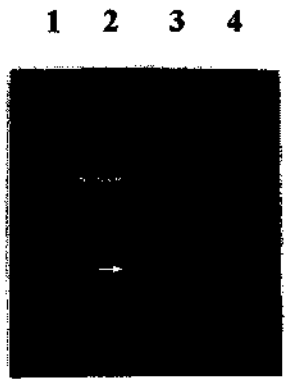

Figure 2. *Taq* 1 digest of 1020 bp fragment from Atlantic DNA using primer set CP113-5' 1\CP113-3' 1

Lane 1 and 4 = 100 bp marker
Lane 2 = undigested 1020 bp fragment
Lane 3 = *Taq* 1 digest of 1020 bp fragment

3.4 Primer set two (CP113-5' 2\CP113-3' 2) and three (CP113-5' 1\CP113-3' 3)

With primer set CP113-5' 2\CP113-3' 2 the expected 760 bp fragment was produced with Maris Piper and Atlantic DNA.

With primer set CP113-5' 1\CP113-3' 3 the expected 700 bp fragment was produced with Maris Piper DNA. The fragment was not strongly produced with Atlantic DNA. This was repeated three times and the result was consistently obtained
(see Figs. 3h and 3i, Appendix 3).

3.5 Conclusions

The CP113 locus specific primer set (CP113-5' 1\CP113-3' 1) produced the expected 1020 bp fragment with DNA from Maris Piper and Atlantic. In addition, a pair of fragments of 530 and 480 bp were produced from Atlantic DNA. The 1020 bp fragment corresponds to alleles *a*, *b* and *d* of the CP113 locus and the 530 and 480 bp fragments correspond to allele *c* (Niewöhner *et al.* 1995). It is therefore assumed that Atlantic possess the *c* allele as well as either the *a*, *b* or *d* allele.

Both Atlantic and Maris Piper are resistant to PCN. Atlantic is thought to be simplex (*Hhhh*) for the dominant *H1* allele (Pineda 1993) and should produce a strong signal for marker allele *a*. Primer sets CP113-5' 2\CP113-3' 2 and CP113-5' 1\CP113-3' 3 are diagnostic for allele *a* and with Atlantic and Maris Piper DNA should produce a similar result. This was not the case. When 'optimised' and tested with DNA from Atlantic, primer set CP113-5' 2\CP113-3' 2 produced a strong signal (see Appendix 3, Fig. 3h, lane 5) whilst primer set CP113-5' 1\CP113-3' 3 produced a week and inconsistent signal (see Appendix 3, Fig. 3i). Due to the inconsistency of primer set CP113-5' 1\CP113-3' 3 we conclude it is unsuitable as a diagnostic tool with Maris Piper and Atlantic.

The *Taq* 1 digest of the 1020 bp fragment is a reliable way of detecting allele *a*, but suffers in that it is extremely time consuming as it involves PCR, gel electrophoresis, fragment purification, a restriction enzyme digest (from a number of duplicated samples) and an other gel electrophoresis. If only a small number of samples require testing, the *Taq* 1 digest could be used to double check the results of using PCR using primer set CP113-5' 2\CP113-3' 2.

4.0 Screening plants with 'known' and 'unknown' response to PCN

4.1 Introduction

Potato lines are presently screened for their response to PCN by nematode. A number of 'advanced selections' developed at IHD-Toolangi have been challenged with nematodes and their resistance status is known. Another group of 'advanced selection' is undergoing nematode challenge and their status is unknown.

4.2 Approach

DNA was isolated from leaf tissue from each of the 10 lines (Table 2) and screened with primer set CP113-5' 1\CP113-3' 1, including a restriction digest with *Taq* 1 and primer set CP113-5' 2\CP113-3' 2. Primer set CP113-5' 1\CP113-3' 3 was not tested.

Population	Line	Status [1]
'Known'	89-12-1	R
	88-102-24	S
	85-51-1	R
	88-85-54	S
	88-59-12	S
'Unknown'	86-2-23	R
	86-34-4	S
	89-27-33	S
	89-88-3	?
	90-105-14	R

Table 2. Status of potato lines to PCN

[1] R = resistant; S = susceptible. Status determined according to nematode challenge with data provided by G. Wilson (IHD-Toolangi). During the project the status of the 'unknown' population became available, except that 89-88-3 was not tested. As the majority of experiments had been done when the status of the material became available it was decided to still use the categories 'known' and 'unknown'

4.3 Results

4.3.1 'Known population with primer set CP113-5' 1\CP113-3' 1, including *Taq* 1 digest

The 1020 bp fragment was generated with DNA from all lines with primer set CP113-5' 1\CP113-3' 1 (Fig. 3) and when digested with *Taq* 1 the 340 bp fragment was produced from lines 88-102-24, 85-51-1, 88-85-54 and 88-59-12 (arrowed) but not 89-12-1 (Fig. 4).

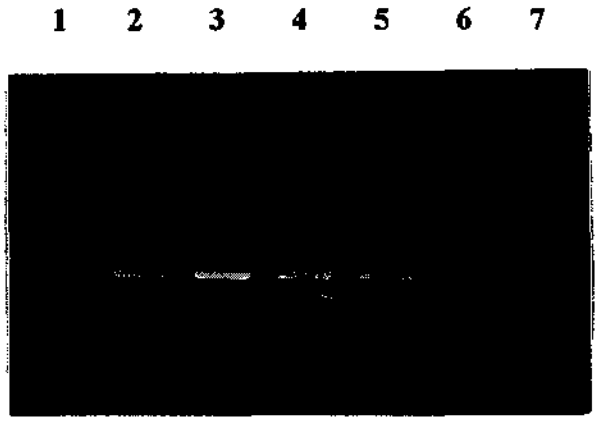

Figure 3. PCR products amplified from 'known' population using primer set CP113-5' 1\CP113-3' 1

Lane 1= 89-12-1
Lane 2 = 88-102-24
Lane 3 = 85-51-1
Lane 4 = 88-85-54
Lane 5 = 88-59-12
Lane 6 = blank
Lane 7 = 1 kb ladder

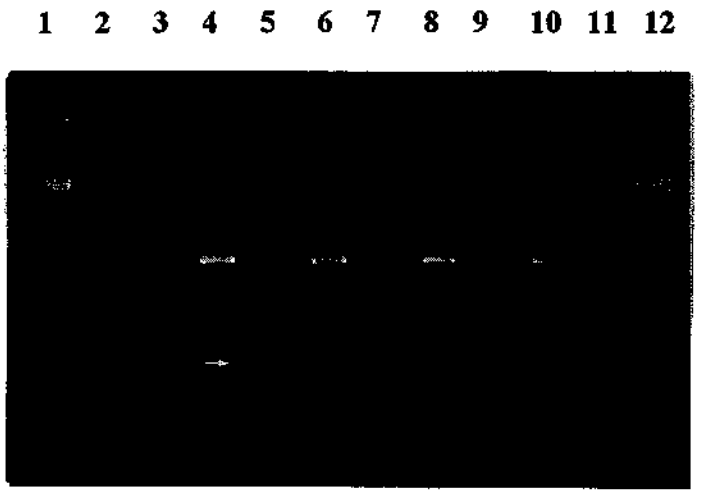

Figure 4. *Taq* 1 digest of 1020 bp fragment from 'known' population using primer set CP113-5' 1\CP113-3' 1

Lanes 1 and 12 = 100 bp marker
Lanes 2 and 3 = 89-12-1 (Lane 2 = undigested; lane 3 = *Taq* 1 digest)
Lanes 4 and 5 = 88-102-24 (Lane 4 = undigested; lane 5 = *Taq* 1 digest)
Lanes 6 and 7 = 85-51-1 (Lane 6 = undigested; lane 7 = *Taq* 1 digest)
Lanes 8 and 9 = 88-85-54 (Lane 8 = undigested; lane 9 = *Taq* 1 digest)
Lanes 10 and 11 = 88-59-12 (Lane 10 = undigested; lane 11 = *Taq* 1 digest)

4.3.2 'Known' population with primer set CP113-5' 2\CP113-3' 2

When screened with primer set CP113-5' 2\CP113-3' 2 DNA from 88-102-24, 88-85-54 and 88-59-12 produced the 760 bp fragment (Fig. 5).

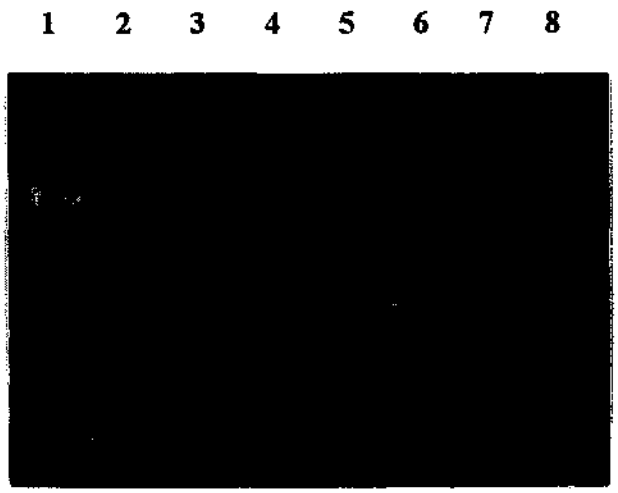

Figure 5. PCR products amplified from 'known' population using primer set CP113-5' 2\CP113-3' 2

Lane 1 = 100 bp marker
Lane 2 = 89-12-1
Lane 3 = 88-102-24
Lane 4 = 85-51-1
Lane 5 = 88-85-54
Lane 6 = 88-59-12
Lane 7 = blank
Lane 8 = 1 kb marker

4.3.3 'Unknown' population with primer set CP113-5' 1\CP113-3' 1, including *Taq* 1 digest

All lines screened with primer set CP113-5' 1\CP113-3' 1 generated the 1020 bp fragment (Fig. 6) and produced the 340 bp fragment (arrowed) when digested with *Taq* 1 (Fig. 7).

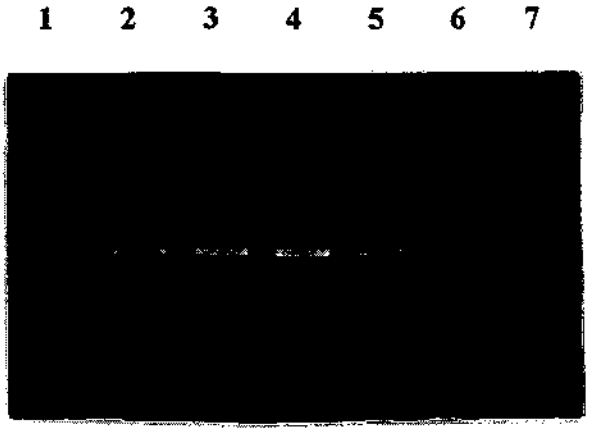

Figure 6. PCR products amplified from 'unknown' population using primer set CP113-5' 1\CP113-3' 1

Lane 1= 86-2-23
Lane 2 = 86-34-4
Lane 3 = 89-27-33
Lane 4 = 89-88-3
Lane 5 = 90-105-14
Lane 6 = blank
Lane 7 = 1 kb marker

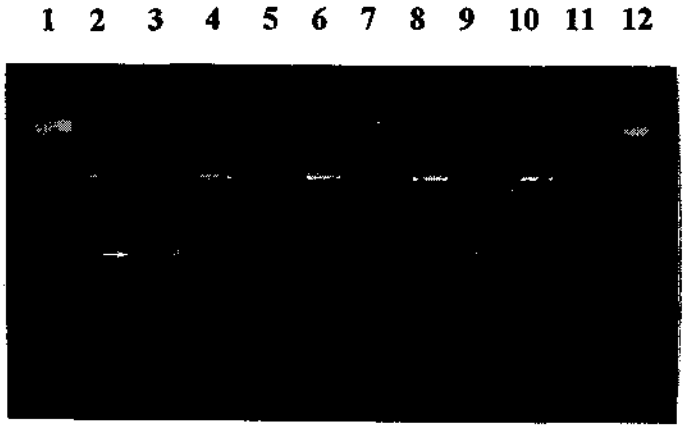

Figure 7. *Taq* 1 digest of 1020 bp fragment from 'unknown' population using primer set CP113-5' 1\CP113-3' 1

Lanes 1 and 12 = 100 bp marker
Lanes 2 and 3 = 86-2-23 (Lane 2 = undigested; lane 3 = *Taq* 1 digest)
Lanes 4 and 5 = 86-34-4 (Lane 4 = undigested; lane 5 = *Taq* 1 digest)
Lanes 6 and 7 = 89-27-33 (Lane 6 = undigested; lane 7 = *Taq* 1 digest)
Lanes 8 and 9 = 89-88-3 (Lane 8 = undigested; lane 9 = *Taq* 1 digest)
Lanes 10 and 11 = 90-105-14 (Lane 10 = undigested; lane 11 = *Taq* 1 digest)

4.3.4 'Unknown' population with primer set CP113-5' 2\CP113-3' 2

When screened with primer set CP113-5' 2\CP113-3' 2 DNA from lines 86-2-23, 89-27-33 and 90-105-14 produced a 760 bp fragment (Fig. 8).

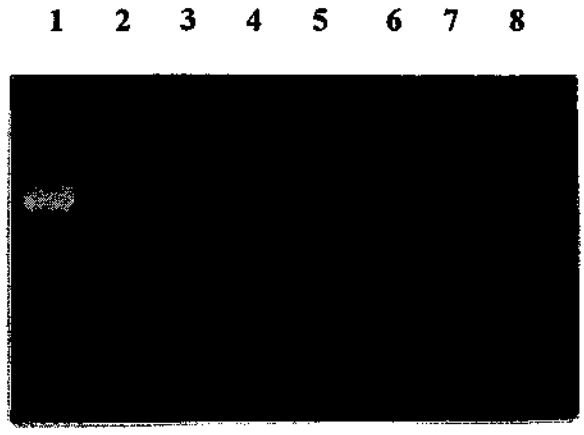

Figure 8. PCR products amplified from 'unknown' population using primer set CP113-5' 2\CP113-3' 2

Lane 1 = 100 bp marker
Lane 2 = 86-2-23
Lane 3 = 86-34-4
Lane 4 = 89-27-33
Lane 5 = 89-88-3
Lane 6 = 90-105-14
Lane 7 = blank
Lane 8 = 1 kb marker

4.4 Conclusions

The advanced selections (with the exception of 89-88-3) have been screened for there response to PCN with a nematode challenge assay and have been designated either resistant or susceptible. In addition, they have been screened with a PCR assay that is diagnostic for the *a* allele. Data from both is summarised in Table 3.

The expectation, if the PCR assay is suitable for PCN resistance screening would be that lines designated from the nematode challenge assay as resistant would also produce the *a* allele specific fragment. This is only the case with two of the lines, namely, 86-2-23 and 90-105-14. With five lines (89-12-1, 88-102-24, 88-85-54, 88-59-12 and 89-27-33)the result from the nematode challenge is the complete opposite of the PCR result. With two lines (85-51-1 and 86-34-4) there is no consistency with the PCR result; where the *Taq* 1 digest assay indicating resistance and primer set CP113-5' 2\CP113-3' 2 indicating susceptibility.

Line	Status [1] (as determined by)		
	Nematode challenge	PCR	
		Taq 1 [2]	5' 2\3' 2 [3]
89-12-1	R	S	S
88-102-24	S	R	R
85-51-1	R	R	S
88-85-54	S	R	R
88-59-12	S	R	R
86-2-23	R	R	R
86-34-4	S	R	S
89-27-33	S	R	R
89-88-3	?	R	S
90-105-14	R	R	R

Table 3. Summary of results obtained from screening 'advanced selections' with nematode challenge and PCR

[1] R = resistant; S = susceptible
[2] *Taq* 1 digest of 1020 bp fragment generated with primer set CP113-5' 1\CP113-3' 1
[3] Primer set CP113-5' 2\CP113-3' 2

5.0 Screening a segregating population

5.1 Introduction

A major benefit of marker assisted selection is the ability to screen large populations of plants at early developmental stages. Marker assisted selection does however, require the linkage to be stable across generations. To test the stability of the linkage, the assay should be tested on a F_1 population that originated from a cross between a resistant and susceptible parent.

The ability to identify the genotype of individuals within a segregating population is extremely important as it can indicate which ones to discard. Identification of individuals within a segregating population is the critical initial step of bulk segregant analysis, a method which allows the identification of markers with increased linkage to the target sequence and subsequently the gene of interest (Michelmore *et al.* 1991).

To determine the suitability of the Niewöhner *et al.* (1995) assay to select plants for resistance to PCN a potato seedling population was screened. The seedlings used were a F_1 population, the product of a cross between the cultivars Rosa (♀ parent, resistant) and Snowgem (♂ parent, susceptible).

5.2 Approach

Hybridisation, seed collection and germination were done at IHD-Toolangi as part of NaPIES. Two expanding leaves from each seedling were collected, one stored at -70 °C (as a reference sample) and the other used for DNA isolation. Following senescence, a minituber was collected from each seedling and stored. The seedlings were individually labelled, as were the stored leaves and tubers. A total of 296 samples were studied.

For the PCR studies primer sets CP113-5' 1\CP113-3' 1 and CP113-5' 2\CP113-3' 2 were used as previously described. A positive result would reveal a 1020 bp fragment with primer set CP113-5' 1\CP113-3' 1, indicating the presence of the *a*, *b* and *d* alleles and with primer set CP113-5' 2\CP113-3' 2 a 760 bp fragment, indicating the presence of the *a* allele. The *Taq* 1 digest was not done as it is technically difficult, time consuming and results in the same information as primer set CP113-5' 2\CP113-3' 2, i.e. the *a* allele.

5.3 Results

Of the 296 seedlings screened with primer set CP113-5' 1\CP113-3' 1, 290 (98%) amplified the 1020 bp fragment as did Rosa and Snowgem. When reacted with primer set CP113-5' 2\CP113-3' 2, 257 (87%) amplified the 760 bp fragment (Table 4), in addition Rosa also amplified the fragment but Snowgem did not.

With both primer set Rosa

Primer set	% of seedlings producing fragment of expected size	Expected fragment size (bp)
CP113-5' 1\CP113-3' 1	98	1020
CP113-5' 2\CP113-3' 2	87	760

Table 4. Response of a seedling population resulting from a cross between Rosa and Snowgem with two primer sets

A representative result is shown in Figs. 9 and 10.

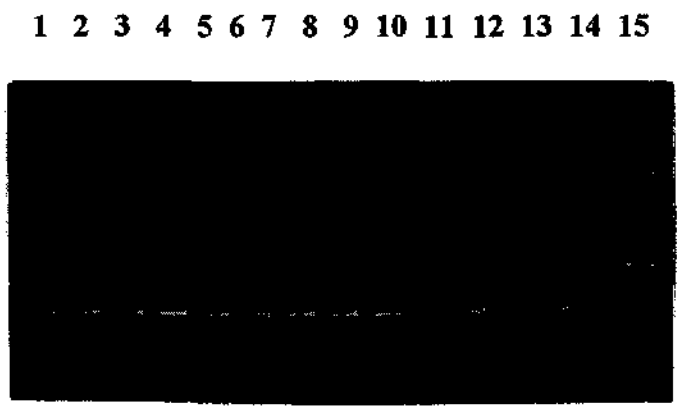

Figure 9. PCR products amplified from 12 individual seedlings of the F_1 population using primer set CP113-5' 1\CP113-3' 1

Lane 1 to 12 = 12 individual seedlings
Lane 13 = Rosa
Lane 14 = blank
Lane 15 = 1 kb marker

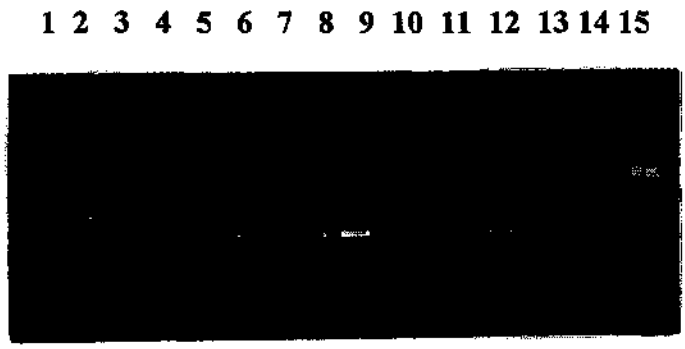

Figure 10. PCR products amplified from 12 individual seedlings of the F_1 population using primer set CP113-5' 2\CP113-3' 2

Lanes 1-12 = 12 individual seedlings
Lane 13 = Rosa
Lane 14 = Snowgem
Lane 15 = 100 bp marker

5.4 Conclusions

As 98 % of the seedlings produced the expected 1020 bp fragment when using primer set CP113-5' 1\CP113-3' 1 this shows that alleles *a*, *b* and *d* are present. It would be expected that 100 % of the seedlings produce this fragment and the six seedlings that did not were tested again where the same result was obtained. Allele *c* was not found in any of the seedlings as the 530 and 480 bp fragments were not amplified.

The primer set CP113-5' 2\CP113-3' 2 revealed that 257 (87 %) of the seedlings had the *a* allele. This is an interesting, but confusing result. The resistant parent used to generate the segregating population was Rosa, which is resistant to PCN. The parentage of Rosa is Wauseon (♀) and J171-B (♂). Wauseon is resistant to PCN (Cunningham *et al.* 1968), the source of resistance which can be traced back three generations to CPC 1673, which is *S. tuberosum* ssp. *andigena*, the original source of the *H1* gene (Toxopeus and Huijsman 1953). It is most likely that Wauseon is simplex (*Hhhh*) for the *H1*. The other parent of Rosa is J171-B, which is a 'andigena clone' and considered susceptible to PCN (G. Wilson, personal communication) and most likely nulliplex (*hhhh*) or possibly simplex if all *S. tuberosum* ssp. *andigena* lines are simplex

Crossing a simplex with a nulliplex would result in a segregation ratio of 1:16 or approximately 20 of the resulting seedlings having *H1*. If J171-B is also simplex for *H1*, the segregation ratio would be 7:16 or approximately 140 seedlings having *H1*. The only way for 87% of the seedling to have *H1* is for both parents to be quadruplex (*HHHH*) or one of them to be triplex. This is most certainly not the case.

These results demonstrate the linkage between CP113 *H1* has been lost or that the marker system approach is not applicable. The only way that the relationship between the two systems can be established is to challenge the seedlings with PCN and relate the result with the marker approach using primer set CP113-5' 2\CP113-3' 2 and the *Taq* 1 digest.

6.0 Effectiveness of CP113 as a for marker for PCN resistance

Marker CP113 was chosen because of;

- its tight linkage with *H1*,
- it mapped to a single locus.

This marker was specifically amplified in the resistant progeny from which the primer sets were designed (Niewöhner *et al.* 1995). The developmental work described here using Atlantic and Maris Piper (Appendix 3 and Section 3) also found that the various primer sets and the *Taq* 1 digest were excellent indicators of the PCN status of the two cultivars.

The finding that potato lines previously screened for there response to PCN do not exhibit a consistent relationship between the markers for *H1* and the result from nematode challenge (Tables 2 and 3) in all cases is most unfortunate, but can be explained.

Firstly, resistant alleles other than *H1* may be present in the potato cultivars and lines used in this study. Momeni *et al.* (1969) reported that other genes (*Fa* and *Fb* = *Gro1*) have been described that also confer hypersensitive resistance to different pathotypes of *G. rostochiensis*. While these genes are thought to have lower expressivity and higher epistatic effects than *H1* (Pineda *et al.* 1993), they may be more widely distributed in the potato lines and cultivars used than in the lines from which the primer sets were designed.

Secondly, recombination between *H1* and CP113 may have occurred early in the breeding history, causing the marker allele CP113 to be non-specific and uninformative. When the cultivars Atlantic and Maris Piper were screened, the markers reacted as predicted. This demonstrates unequivocally that the linkage between *H1* and CP113 is present.

Thirdly, major genes for resistance tend to be accompanied by minor gene complexes (Ross 1986). During several backcrosses, the gene complex is split up and the residual resistance that remains is always lower than the original wild source.

The problems we have found with the primer sets have been found by others (Peleman 1997).

7.0 An alternative approach - Part 1

7.1 Introduction

During the course of this project we became aware of another study that identified an additional marker linked to *H1* (Pineda *et al.* 1993). To obtain this marker, two segregating populations were used. Firstly, a dihaploid population derived via parthenogenesis from Atlantic and thought to be simplex (*Hhhh*) for the *H1* allele. The other was a tetraploid population resulting from a cross between Steuben (♀ parent, resistant and thought to be simplex (*Hhhh*) for the *H1* allele) and Monona (♂ parent, susceptible).

Seedlings were challenged with nematodes and bulk segregant analysis (Michelmore *et al.* 1991) used. DNA was pooled, digested with five different restriction enzymes, separated with gel electrophoresis, transferred to nylon membrane. The nylon membranes were probed with 60 different cDNA and genomic clones from a tomato\potato RFLP map (Bonierbale *et al.* 1988; Tanksley *et al.* 1992). Of the probes screened, one designated CD78, was found to be tightly linked with the *H1*. When DNA from the Atlantic population was digested with *Eco* R1 and probed with CD78 resistant lines produced two bands at about 9 and 5.5 kb, whereas susceptible lines only produced a band at 5.5 kb. The sequence of CD78 has not been determined, therefore it was not possible to design primers and use a PCR based approach.

7.2 Approach

DNA was isolated by using a scaled up version of Fulton *et al.* (1995) as described in Appendix 2. DNA samples were treated with RNAase (to degrade any RNA present), quantified fluormetrically and stored at -20 °C. For each sample, 11 µg of DNA was digested with *Eco* R1, *Hind* III or *Xba* 1 (Promega) according to the manufacturers instructions and separated in a 1% agarose gel in x1 TAE. DNA was transferred to Hybond N^+ membrane (Amersham Life Sciences) using x20 SSC and cross linked by placing the filter, DNA side down on a UV transilluminator for 5 minutes (Sambrook *et al.* 1989). Only data from DNA digested with *Eco* R1 is presented.

The CD78 probe was supplied by Dr S. Tanksley (Cornell University, USA) as a lyophilised fragment 550 bp long. This was ligated into pUC8 within the *Pst* 1 cloning site and transformed into competent TGI cells using standard techniques (resulting in p CD78 x TGI) and grown overnight in LB medium with 100 µg mL^{-1} ampicillin at 37 °C. The pCD78 was isolated and the 550 bp CD78 fragment out using *Pst* 1, separated with gel electrophoresis using low melting temperature agarose and purified from the gel.

Purified CD78 was fluormetrically labelled (dUTP) with nick translation using a FluoroGreen ™ kit (Amersham Life Sciences).

The nylon membrane was pre-hybridised in Rapid-hyb buffer (Amersham Life Sciences) for 2 hours at 65 °C. The labelled probe and membrane were hybridised overnight at 65 °C and the membrane washed with x1 SSC, 0.1% SDS and then x 0.1 SSC, 0.1% SDS. Both washes were at 65 °C for 15 minutes.

After hybridisation the Green Images CDP-*Star* detection module (Amersham Life Sciences) was used to detect the FluoroGreen labelled CD78. The result was visualised by exposing the filter to Kodak X-Omat AR film for 15 minutes and developing according to the manufacturers instructions.

7.3 Results

7.3.1 Cultivars Atlantic, Rosa and Snowgem

DNA from Rosa, Snowgem and Atlantic was digested to completion with *Eco* R1 (Fig. 11a) and each cultivar produced a strong hybridisation signal at about 5.5 and 6 kb when hybridised with CD78. A faint band (arrowed) at about 9 kb was produced with Snowgem (Fig. 11b).

Figure 11. Analysis of potato cultivars probed with CD78

11a contains DNA digested with *Eco* R1 and 11b is the same DNA probed with CD78

Lane 1 and 4 = Rosa
Lane 2 and 5 = Atlantic
Lane 3 and 6 = Snowgem

7.3.2 'Known' and 'Unknown' populations

DNA from the 'known' and 'unknown' populations (see section 4) was digested to completion with *Eco* R1. A range of hybridisation patterns were obtained (Fig. 12), however only scorable bands were obtained in six of the 10 lines screened. Of the six scorable cultivars, all had a common band at 6 kb, except 90-105-14. Of the line known to be PCN resistant which produced scorable bands, all had a common band at 9 kb. These results are summarised in Table 5.

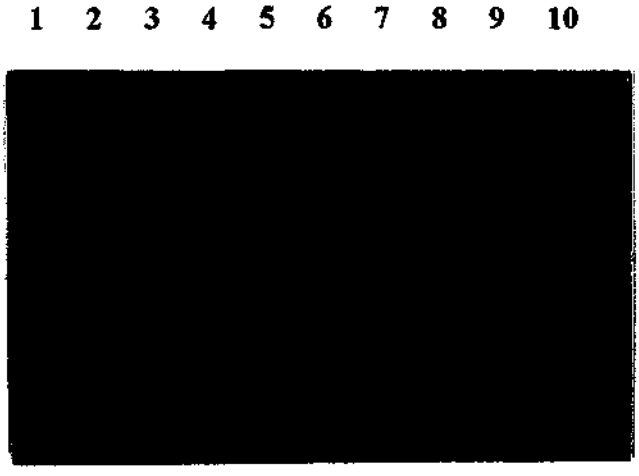

Figure 12. Analysis of 'known' and 'unknown' population probed with CD78

Lane 1 = 89-12-1

Lane 2 = 88-102-24

Lane 3 = 85-51-1

Lane 4 = 88-85-54

Lane 5 = 88-59-12

Lane 6 = 86-2-23

Lane 7 = 86-34-4

Lane 8 = 89-27-33

Lane 9 = 89-88-3

Lane 10 = 90-105-14

Line		Status	Banding pattern	
			No. of fragments	Fragment size (kb)
'Known'	89-12-1	R	-	-
	88-102-24	S	2	6, 5.5
	85-51-1	R	3	9, 6, 5.5
	88-85-54	S	-	-
	88-59-12	S	1	6
'Unknown'	86-2-23	R	2	9,
	86-34-4	S	-	-
	89-27-33	S	-	-
	89-88-3	?	1	6
	90-105-14	R	1	9

Table 5. Banding pattern resulting from probing DNA from 'known and 'unknown' populations with CD78

7.4 Conclusions

The results obtained when screening plants with a DNA probe do not consistently relate with those obtained with nematode challenge. The cultivars Atlantic and Rosa are both resistant to PCN and both have a 5.5 and 6 kb, whereas Snowgem which is susceptible has an additional band at 9 kb. It is tempting to consider that this additional band maybe associated with susceptibility. When the banding pattern of the six cultivars from the 'known' and 'unknown' populations are compared to the results with nematode challenge this relationship is not present. Indeed the reverse is true. The lines 85-51-1, 86-2-23 and 90-105-14 which are resistant to PCN all have a common band at 9 kb.

It is premature to be dismissive of this approach. The CD78 probe has only been tested with relatively few cultivars and the results from only one restriction enzyme (*Eco* R1) evaluated. It is most likely that by different restriction enzymes and hence a different restriction pattern a constant relationship may be obtained. Consideration should also be given to obtaining the sequence of CD78.

An alternative approach - Part 2

7.5 Introduction

The molecular marker system being used with the CP113 series of primers result from the conversion of the RFLP marker (CP113) to a PCR based system by obtaining sequence data. As mentioned earlier, the marker CD78 had not been sequenced, therefore it was necessary to use Southern analysis, which suffers in that it is labour intensive. Molecular markers have also been developed using RAPDs (Young 1994; Paterson 1996). For this series of experiments the approach was to screen potato DNA with two primers, one constantly used and the other of variable sequence, with the intent of finding a banding pattern that was unique for the cultivar. As one of the primers used is associated with CP113, the expectation being that the variable primer may be binding to a portion of the genome that is associated with a differential response to PCN.

It is important to stress that this series of experiments were done to ascertain the suitability of the approach and not to generate a marker associated with PCN resistance.

7.6 Approach

DNA from Rosa and Snowgem was screened with primer CP113-5' 1 (constant) and 20 different 10-mer random primers (Appendix 1d). For any primer combination four reactions were done; each DNA with only the random primer and each DNA with the random primer and CP113- 5' 1.

7.7 Results

DNA was amplified with most of the combinations of CP113-5' 1 and the random primers tested. Many of the primers did not result in much polymorphism, with the DNA profiles for Rosa and Snowgem being similar, for example primer 2-09 (Fig. 13).

Four of the random primers used resulted in polymorphisms where the DNA profiles generated by Rosa and Snowgem were sufficiently different, for example primer 2-17 (Fig. 14).

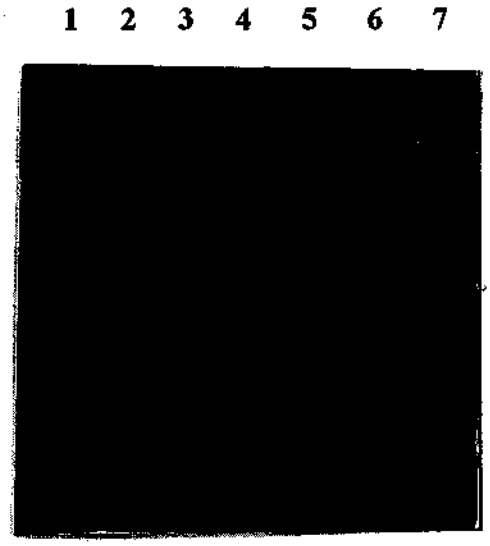

Figure 13. PCR products amplified from Rosa and Snowgem using primers CP113-5' 1 and 2-09

Lane 1 = Rosa with primer 2-09 only
Lane 2 = Snowgem with primer 2-09 only
Lane 3 = Rosa with both primers
Lane 4 = Snowgem with both primers
Lane 5 = blank
Lane 6 = *Eco* R1 digested SPP-1 bacteriophage DNA (marker)
Lane 7 = *Hind* III λ phage DNA (marker)

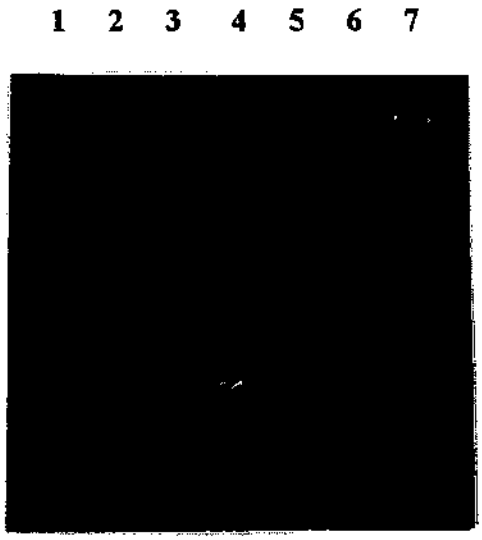

Figure 14. PCR products amplified from Rosa and Snowgem using primers CP113-5' 1 and 2-17

Lanes as above except that primer 2-17 used

7.8 Discussion

RAPDs have the potential to develop a molecular marker system for PCN as polymorphisms can be detected. The approach of using a primer from the CP113 locus (CP113-5' 1) and a random primer, while not essential, is more likely to amplify DNA associated with a suitable marker.

While using RAPDs is a suitable approach, it cannot be readily used at present. As PCN is a quarantinable disease in Australia it is not possible to screen a segregating population with nematode challenge and use bulk segregant analysis. One of the expectations of this project was that if the primers associated with the CP113 marker were diagnostic for the *a* allele then seedlings could be screened and those susceptible to PCN could be discarded.

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8.0 Recommendations

8.1 Extension and adoption by industry

The results from this research describe the evaluation of a number of approaches to develop a diagnostic tool based on molecular markers that could be applied to screen germplasm for its response to PCN.

In its present form the results cannot be utilised directly by industry. Industry adoption will occur, in the longer term, through the refinement of the system, its incorporation into the NaPIES programme and the release of PCN resistant cultivars.

8.2 Direction for future research

One of the expectations of this research was that a rapid and reliable system would be developed to screen potato germplasm for its response to PCN. Such a system would alleviate the necessity to sent plants abroad for challenge with nematodes.

The marker system based on using primers associated with CP113 is not suitable as a diagnostic tool to screen for PCN resistance. This does not mean that molecular markers are an inappropriate method, but simply that other approaches need to be developed.

Further research using the CD78 probe is warranted, as it has been shown to be effective for screening the progeny from tetraploid populations. As a DNA probe it suffers because it is necessary to do DNA hybridisation which is time consuming and relatively few samples can be processed at any one time. Results would be generated more quickly if the CD78 marker were sequenced and a PCR test developed.

The use of RAPDs is an alternative approach but its use is limited. Markers could be developed, but it would be necessary to have a segregating population that has previously screened by nematode challenge.

Alternative approaches based on Amplified Fragment Length Polymorphisms (AFLPs) are being developed for potatoes which are being used to develop markers for a diversity of attributes including PCN.

Further funding for developing molecular markers for PCN should only be considered if PCN resistance breeding becomes a priority in NaPIES and not an objective.

8.3 Financial/commercial benefits of adoption of research findings

These will only occur when a marker system is integrated into the conventional breeding programme.

Appendix 1a. Primer sequences

Primer	Sequence
CP113-5' 1	CTT ACA GTC GCC GTA T
CP113-5' 2	GCC TTA CAG TCG CCG TAT
CP113-3' 1	AAA TGG GGC AAT CTG A
CP113-3' 2	GTT GAA GAA ATA TGG AAT CAA A
CP113-3' 3	GTG AGA TAT ACG AGA AAT T

Appendix 1b. Annealing temperatures and cycle number for the primer sets used

Primer set	Annealing temperature (°C)	Cycle number	Number of cycles
CP113-5' 1\CP113-3' 1	45	1-35	35
CP113-5' 2\CP113-3' 2 [1]	60 58	1-5 6-30	30
CP113-5' 1\CP113-3' 3 [1]	49 47	1-5 6-30	30

[1] Primer sets CP113-5' 2\CP113-3' and CP113-5' 1\CP113-3' have two annealing temperatures. The first temperature is higher to ensure that the initial primer-template hybridisation events involve only those reactants with the greatest complimentarily. The second temperature is lower, but because the target amplicon has already begun amplification, it out competes any non-specific products.

Appendix 1c. Predicted response for the various primer sets tested

Primer set	Allele	Fragment sizes (bp)
CP113-5' 1\CP113-3' 1	*a*, *b* and *d*	1020
	c	530 and 480
	allele *a* can be distinguished from *b*, *c* and *d* with a *Taq* 1 digest of the 1020 bp fragment resulting in a fragment of 340 bp	
CP113-5' 2\CP113-3'	*a*	760
CP113-5' 1\CP113-3'	*a*	700

Appendix 1d. Primers used to generate RAPDs

Primer	Sequence (5' → 3')
2-01	AAG CTG CGA G
2-02	CAC GGC GAG T
2-03	CTG GCG TGA C
2-04	GGG TAA CGC C
2-05	CAA TCG CCG T
2-06	TTC GAG CCA G
2-07	GAA CGG ACT C
2-08	GTC CCG ACG A
2-09	TGT CAT CCC C
2-10	GGT GAT CAG G
2-11	CCG AAT TCC C
2-12	GGC TGC AGA A
2-13	CTG ACC AGC C
2-14	GTA CCG TGG T
2-15	CCA CAC TAC C
2-16	CAC CCG GAT G
2-17	CAC AGG CGG A
2-18	TGA CCC GCC T
2-19	GGA CGG CGT T
2-20	TGG CGC ACT G

Appendix 2. DNA isolation

Introduction

The extraction of DNA from plant tissue is a critical step in molecular marker studies. All extraction methods start with some form of tissue maceration and cell lysis, followed by the recovery and purification of DNA. Different isolation methods can have a dramatic effect on the quality, purity and molecular weight of the DNA obtained. Generally, time consuming methods, requiring large amounts of plant tissue are used to produce DNA of high quality and high molecular weight (e.g. Dellaporta *et al.* 1983). Such methods have limited application for molecular marker based breeding programmes where large numbers of plant samples need to be analysed in a short period of time. Preferred methods for DNA isolation should be rapid, simple and reliable, requiring only a small amount of tissue, be non-destructive to the plant with the resulting DNA being pure and be able to be stored for extended periods or used directly for PCR.

A variety of methods have been developed for the rapid extraction of DNA (e.g. Edwards *et al.* 1991; Thomson and Dietzgen 1995 and Fulton *et al.* 1995) and some have been developed commercially and are available as kits, for example 'GeneReleaser' (Bio Ventures Inc., Tennessee; Dawson *et al.* 1995). These methods have been used to isolate plant DNA, but have not been evaluated for potato.

Methods

DNA was isolated from glasshouse grown plants of Atlantic or Maris Piper using five different methods, one detailed and time consuming and the other four being rapid.

The method of Dellaporta *et al.* (1983) is time consuming but produces high quality DNA and was used for comparison to the other methods.

Edwards *et al.* (1991) was originally used to isolate DNA from *Brassica napus* and has the advantage of not requiring phenol:chloroform extractions.

A simple, single-step plant tissue preparation method was developed by Thomson and Dietzgen (1995) for detecting viruses by PCR. The basic method of boiling a small amount of leaf tissue (*ca.* 0.5 cm^2) in a Tris, K Cl and EDTA buffer was tested with a small number of modifications;

i. to prevent evaporation of the buffer during boiling, material was processed in a microwave oven with a mineral oil overlay,

ii. plant tissue was ground with a disposable pestle in extraction buffer,

iii. after boiling, the DNA was precipitated with Na acetate and ethanol.

The method of Fulton *et al.* (1995) was originally developed for the preparation of tomato DNA and involves a chloroform extraction. The original method was slightly modified by using a small amount of acid washed sand during tissue grinding and adding an extra chloroform extraction.

'GeneReleaser' is a proprietary reagent originally developed for the release of DNA from whole blood and bacterial colonies. Bio Ventures Inc. have modified the procedure for plants and this was used according to the manufacturers instructions.

The quality of DNA was checked by using ethidium bromide stained agarose gels, initially after isolation and after storage at -20 °C for four weeks.

Results and Discussion

Good quality, high molecular weight DNA was isolated with the method of Dellaporta *et al.* (1983). The DNA was stable after storage for four weeks (Fig. 2a). The main disadvantages of this method are that only eight samples can be easily processed at any one time and the method requires a full day to complete.

The method of Edwards *et al.* (1991), the modifications of Thomson and Dietzen (1995) and the 'GeneReleaser' kit resulted in only small yields of low quality DNA that rapidly degraded. As the ability to store DNA is essential, particularly for re-screening samples these methods were considered not suitable and not pursued further.

The slight modification of the Fulton *et al.* (1995) method resulted in good quality, high molecular weight DNA that was stable after storage at -20 °C for four weeks (Fig. 2b). From a 0.5 cm^2 piece of leaf tissue, sufficient DNA can be obtained for about 50 PCR reactions. The method has the distinct advantage that 24 samples can be processed in about 4 hours and became the method of choice for all further work.

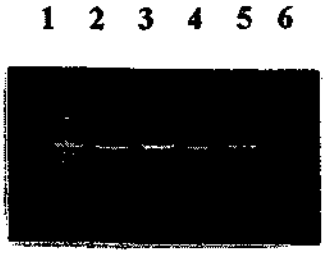

Figure 2a. Potato DNA isolated using the method of Dellaporta *et al.* (1983) and stored for four weeks at -20 °C

Lane 1 = *Hind* III λ phage DNA (marker)
Lane 2 and 3 = 500 ng Maris Piper DNA
Lane 4 and 5 = 500 ng Atlantic DNA
Lane 6 = blank

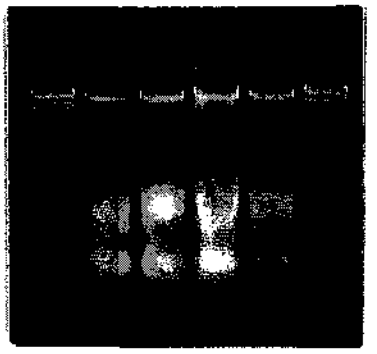

Figure 2b. Potato DNA isolated using the modified method of Fulton *et al.* (1995) and stored for four weeks at -20 °C

Lane 1 = *Hind* III λ phage DNA (marker)
Lane 2 and 3 = 500 ng Maris Piper DNA
Lane 4 and 5 = 500 ng Atlantic DNA

Appendix 3. Development of favourable conditions for identification of RFLP locus CP113 and *a* allele

Introduction

To assess the suitability of the assay developed by Niewöhner *et al.* (1995) the cultivars Atlantic and Maris Piper were used as they are known sources of the *H1* gene (Gebhardt *et al.* 1993; Pineda *et al.* 1993).

A range of variables have been tested, including the concentration of template DNA and Mg, the type and concentration of polymerase. Also evaluated have been the detergents Triton X-100 and Tween 20, which can improve polymerase stability, increase hybridisation stringency and\or greatly improve the sensitivity, specificity and yield. To prevent polymerisation of misprimed sequences prior to thermal cycling, the physical separation of polymerase and template has been tested using a polymerase antibody (Kellog *et al.* 1994). Annealing temperature is also an important variable but has been previously optimised (Niewöhner *et al* 1995) and detailed in Table 1b.

Methods

General

Leaf tissue of the cultivars Atlantic and Maris Piper were sourced from the IHD-Toolangi and genomic DNA was isolated using a slightly modified version of Fulton *et al.* (1993) and quantified fluorometrically (Hoefer TKO 100).

To prevent contamination and maintain reproducibility, amplification mixes were prepared on ice in a laminar flow cabinet using sterile pipette tips with filters. Primers and dNTPs were aliquoted to small volumes to prevent any contamination resulting from continual thawing and freezing. The reaction conditions used for each experiment are detailed.

All PCR reactions were done in thin walled tubes in a 50 µL volume mix using a FTS-960 thermal cycler (Corbett Research). The cycling conditions were: denaturation at 93 °C, initially for 3 minutes and for 30 seconds in all subsequent cycles, primer annealing for 45 seconds at the specified temperature for each primer set (see Table 1b) and extension for 72 °C for 90 seconds and 10 minutes in the last cycle.

PCR products were resolved by gel electrophoresis through 1% (w\v) agarose in x1 TAE CONTAINING 0.5 µg mL^{-1} ethidium bromide. Molecular size standards of either 100 bp or 1 kb (Pharmacia Biotech) or occasionally *Hind* III λ phage DNA or *Eco* R1 digested SPP-1 bacteriophage DNA (Bresatec Ltd) and control reactions comprising all reagents except template DNA were run with all samples. Gels were photographed over UV light using a Polaroid MP4 camera with 667 film (300 ASA) (Sambrook *et al.* 1989).

Development of PCR with primer set CP113-5' 1\CP113-3' 1

The experiments that follow are presented in a standard format. The concentration of the variable (s) is listed with the concentration of the other components of the PCR reaction mix.

For each experiment a 100 bp and\or 1 kb marker was used to determine the size of the PCR generated fragments.

All experiments contained a blank in which no DNA was present. The majority of experiments were repeated at least two times.

Effect of Mg concentration

The Mg concentration in the reaction mix can be varied, usually within the range of 0.5 to 5 mM. Determining a suitable Mg concentration is important as it has an affect on polymerase activity and incorporation of dNTPs which can influence the specificity and yield of a PCR product.

Mg concentration	**1, 2 3 and 4 mM**
DNA concentration	100 ng
Primer concentration	0.25 µM
dNTPs	100 µM
Polymerase type and concentration	Ampli*Taq* 1 U
Buffer	10 mM Tris, 50 mM KCl (pH 8.3)

The CP113 locus specific fragment of 1020 bp (arrowed) was produced from Atlantic DNA at 2 to 4 mM $MgCl_2$, with slightly more product at 3 and 4 mM (Fig. 3a). The two less intense bands below the 1020 bp fragment possibly result from additional copies of the CP113 locus. This has been observed in other cultivars and diploid material. (Gebhardt, personal communication). There is less background amplification at 3 mM Mg than 4 mM. Amplification did not occur at 1 mM Mg.

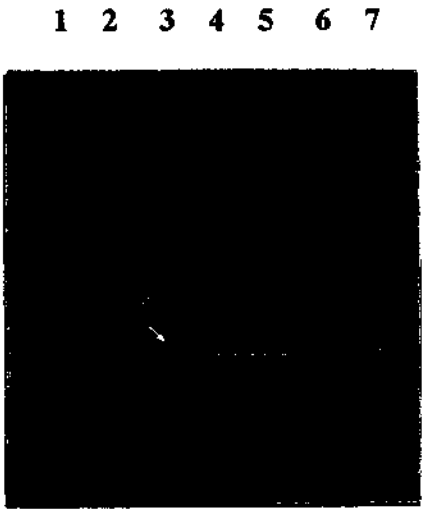

Figure 3a. Effect of $MgCl_2$ concentration on amplification of Atlantic DNA using primer set CP113-5' 1\CP113-3' 1. The 1020 bp fragment is indicated

Lane 1 = 100 bp marker
Lane 2 = 1 mM $MgCl_2$
Lane 3 = 2 mM $MgCl_2$
Lane 4 = 3 mM $MgCl_2$
Lane 5 = 4 mM $MgCl_2$
Lane 6 = blank
Lane 7 = 1 kb marker

Effect of DNA concentration

The DNA concentration used can be important, depending on its source and what the PCR is being used for. When PCR is being used to generate DNA fingerprints its concentration can have a profound effect.

DNA concentration	50, 75, 100 and 125 ng
Mg concentration	3 mM
Primer concentration	0.25 µM
dNTPs	100 µM
Polymerase type and concentration	Ampli*Taq* 1U
Buffer	10 mM Tris, 50 mM KCl (pH 8.3)

The CP113 locus specific fragment of 1020 bp was produced from all of the DNA concentrations tested with Atlantic and Maris Piper (Fig. 3b). As with the previous experiment additional bands were produced with Atlantic. These were not present with Maris Piper DNA.

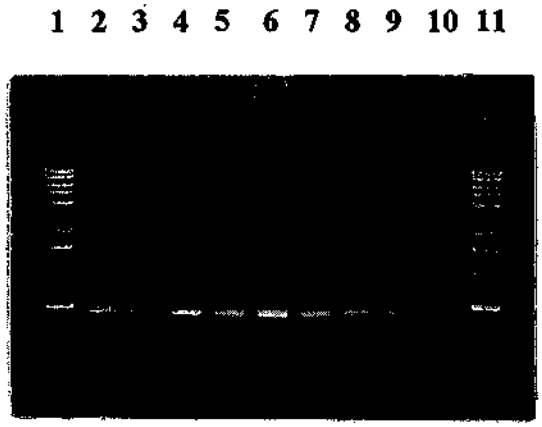

Figure 3b. Effect of DNA concentration on amplification of Atlantic and Maris Piper DNA using primer set CP113-5' 1\CP113-3' 1

Lanes 1 and 11 = 1 kb marker
Lanes 2 and 3 = 50 ng DNA (lane 2 = Maris Piper; lane 3 = Atlantic)
Lanes 4 and 5 = 50 ng DNA (lane 4 = Maris Piper; lane 5 = Atlantic)
Lanes 6 and 7 = 50 ng DNA (lane 6 = Maris Piper; lane 7 = Atlantic)
Lanes 8 and 9 = 50 ng DNA (lane 8 = Maris Piper; lane 9 = Atlantic)
Lane 10 = blank

Effect of polymerase type and Mg concentration

The introduction of thermostable polymerases for PCR was one of the major technological advances that allowed the cycling to be automated. Most polymerases used for PCR are derived from *Thermus aquaticus* and commonly referred to as 'Taq', although other sources are commercially available.

Polymerase type	Polymerase concentration (U) [5]	Mg concentration
Stoffel fragment [1]	5	1, 2 and 3 mM
Red Hot [2]	0.5	1, 2 and 3 mM
Dynazyme [3]	1	not tested
Ampli*Taq* [4]	1	3 mM

[1] A modified version of the recombinant Ampli*Taq* DNA polymerase which has a deletion of 289 amino acids from the N terminus. This version has no intrinsic 5' to 3' exonuclease activity and the polymerase exhibits optimal activity over a broad range of Mg concentrations. Manufactured by Perkin Elmer (UK)

[2] Derived form *T. icelandicus* and has 5' to 3' exonuclease activity. Manufactured by Advanced Biotechnologies Ltd (UK)

[3] Derived from *T. brockianus* and has 5' to 3' exonuclease activity. Optimised for Mg concentration. Manufactured by Finnzymes Oy (Finland)

[4] A recombinant polymerase derived from *T. aquaticus* and has 5' 3' exonuclease activity. Manufactured by Perkin Elmer (UK)

[5] Manufacturers recommendation

DNA concentration	100 ng
Primer concentration	0.25 µM
dNTPs	100 µM

All polymerases produced amplification products, some of which were non-specific (Fig. 3c). Stoffel fragment was inactive at 1 mM Mg (lane 2), and produced non-specific fragments at 2 and 3 mM Mg (lanes 3 and 4). Red Hot was inactive at 1 mM Mg (lane 5), produced the CP113 locus specific fragment of 1020 bp at 2 mM Mg (lane 6) and at 3 mM Mg produced only weak signals (lane 7). Dynazyme produced a very weak signal for the 1020 bp fragment (lane 8) and Ampli*Taq* produced the CP113 locus fragment (lane 9).

This experiment was repeated and Ampli*Taq* was found to be the most reproducible polymerase.

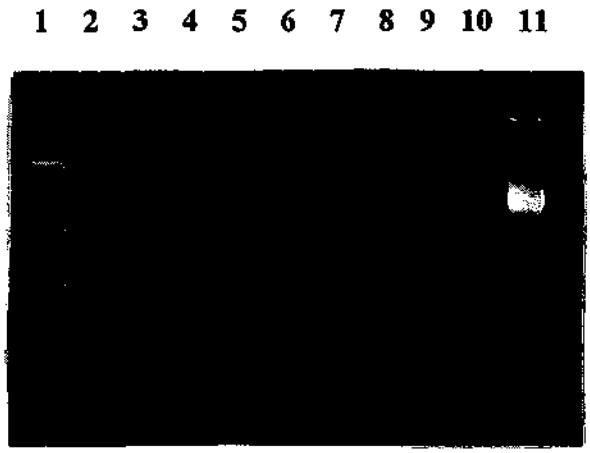

Figure 3c. Effect of four polymerases and $MgCl_2$ concentration on amplification of Atlantic DNA using primer set CP113-5' 1\CP113-3' 1

Lane 1= 1 kb marker
Lanes 2, 3 and 4 = Stoffel fragment (lane 2 = 1 mM Mg, 3 = 2 mM Mg and 4 = 3 mM Mg)
Lanes 5, 6 and 7 = Red Hot (lane 5 = 1 mM Mg, 6 = 2 mM Mg and 7 = 3 mM Mg)
Lane 8 = Dynazyme
Lane 9 = Ampli*Taq*
Lane 10 = blank
Lane 11 = 100 bp marker

Effect of Ampli*Taq* concentration

As polymerase is one of the most expensive reagents used, there are economic advantages in being able to use the least possible concentration and still obtain a reproducible result.

Ampli*Taq*	**0.5, 0.75 and 1 U**
DNA concentration	100 ng
Mg concentration	3 mM
Primer concentration	0.25 µM
dNTPs	100 µM
Buffer	10 mM Tris, 50 mM KCl (pH 8.3)

Ampli*Taq* at 0.5 and 0.75 U were not particularly effective for amplifying DNA from Atlantic or Maris Piper (Fig. 3d; lanes 2 to 5). The CP113 locus specific fragment of 1020 bp was produced from Atlantic and Maris Piper DNA with 1 U of Ampli*Taq* (lanes 6 and 7).

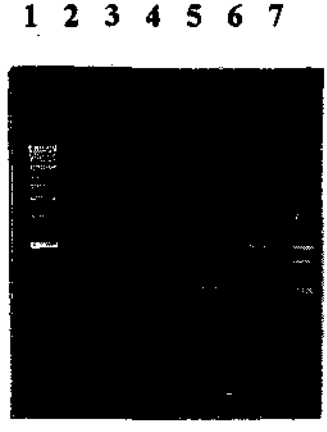

Figure 3d. Effect of Ampli*Taq* concentration on amplification of Maris Piper and Atlantic DNA using primer set CP113-5' 1\CP113-3' 1

Lane 1 = 1 kb marker
Lane 2 and 3 = 0.5 U Ampli*Taq* (lane 2 = Maris Piper; lane 3 = Atlantic)
Lane 4 and 5 = 0.5 U Ampli*Taq* (lane 4 = Maris Piper; lane 5 = Atlantic)
Lane 6 and 7 = 0.5 U Ampli*Taq* (lane 6 = Maris Piper; lane 7 = Atlantic)

Effect of detergent type and concentration

Some ionic detergents such as sodium dodecyl sulphate, which are used during DNA isolation can inhibit polymerase activity. This inhibitory affect can be reversed by some non-ionic detergents such as Tween 20.

Detergent type	Concentration
Tween 20	0.1 and 0.2%
Triton X-100	0.1 and 0.2%

DNA concentration	100 ng
Mg concentration	3 mM
Primer concentration	0.25 µM
dNTPs	100 µM
Polymerase type and concentration	Ampli*Taq* 1 U
Buffer	10 mM Tris, 50 mM KCl (pH 8.3)

The CP113 locus specific fragment of 1020 bp was produced from Maris Piper and Atlantic DNA without the addition of any detergent (Fig. 3e, lanes 2 and 3). Adding Tween 20 slightly decreased the product yield (lanes 5 and 6) and Triton X-100 had an inhibitory effect (lanes 8 and 9). A combination of both detergents also had an inhibitory effect (lanes 11 and 12).

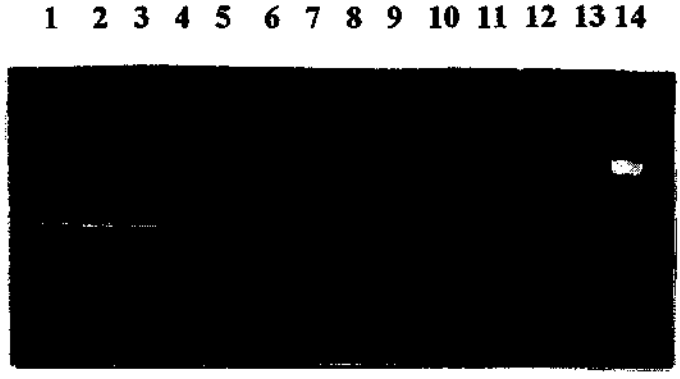

Figure 3e. Effect of Tween 20 and Triton X-100 on amplification of Maris Piper and Atlantic DNA using primer set CP113-5' 1\CP113-3' 1

Lane 1 = 1 kb marker
Lanes 2 and 3 = no detergent (lane 2 = Maris Piper, lane 3 = Atlantic)
Lane 4 = blank
Lane 5 and 6 = 0.1% Tween 20 (lane 5 = Maris Piper; lane 6 = Atlantic)
Lane 7 = blank with 0.1% Tween 20
Lanes 8 and 9 = 0.2% Triton X-100 (lane 8 = Maris Piper; lane 9 = Atlantic)
Lane 10 = blank with 0.2% Triton X-100
Lanes 11 and 12 = Tween 20 and Triton X-100 (lane 11 = Maris Piper; lane 12 = Atlantic)
Lane 13 = blank with Tween 20 and Triton X-100
Lane 14 = 100 bp marker

Effect of TaqStart ™ antibody

The yield and specificity of PCR can be improved by using the 'hot start' method. This involves withholding an essential component (e.g. polymerase or primers) from the reaction mix until all other components have been heated to above the annealing temperature. The missing component is then added, either manually, or by physical separation using for example a wax barrier. The 'hot start' method while effective, is cumbersome and time consuming. Recently, a new and elegant variation on the 'hot start' was developed, which makes use of a monoclonal antibody that binds to and inhibits the polymerase when preparing the reaction mix. During the first heating cycle, the antibody\polymerase complex dissociates and the antibody is inactivated, restoring the polymerase activity.

TaqStart™	Used according to the manufacturers recommendation (Clontech Inc. USA)

DNA concentration	100 ng
Mg concentration	3 mM
Primer concentration	0.25 µM
dNTPs	100 µM
Polymerase type and concentration	Ampli*Taq* 1 U
Buffer	10 mM Tris, 50 mM KCl (pH 8.3)

Incorporating the TaqStart antibody decreased the yield of the 1020 bp fragment (Fig. 3f, lanes 2 and 3) compared to Ampli*Taq* alone (lanes 5 and 6).

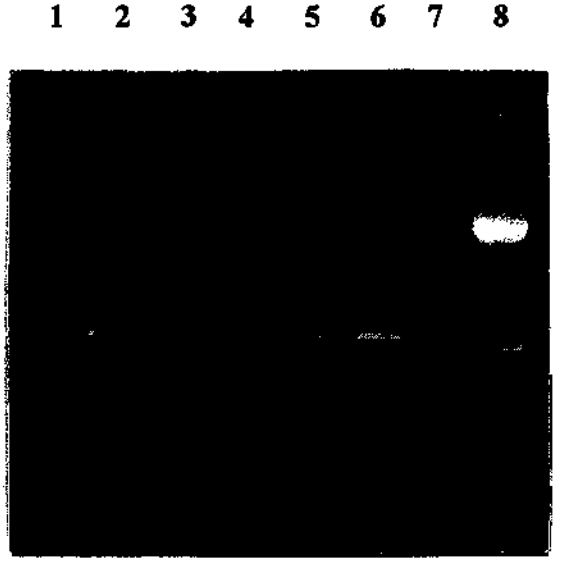

Figure 3f. Effect of TaqStart antibody on amplification of Maris Piper and Atlantic DNA using primer set CP113-5' 1\CP113-3' 1

Lane 1 = 1 kb marker
Lanes 2 and 3 = with TaqStart antibody (lane 2 = Maris Piper; lane 3 = Atlantic)
Lane 4 = blank with TaqStart antibody
Lanes 5 and 6 = without TaqStart antibody (lane 5 = Maris Piper; lane 6 = Atlantic)
Lane 7 = blank
Lane 8 100 bp marker

Optimisation of *Taq* 1 digest

The 1020 bp fragment was generated from Atlantic DNA using the primer set CP113-5' 1\CP113-3' 1 and the conditions as detailed in Table 1 (Section 3.0). Five 50 µL reactions were prepared and the PCR products from four reactions pooled and the DNA concentrated by ethanol precipitation (Sambrook *et al.* 1989). The concentrated solution of 1020 bp fragments was digested with 10 U of *Taq* 1 (Promega) according to the manufacturers instructions. The restriction enzyme digest products were resolved by gel electrophoresis.

The expected 340 bp restriction fragment (arrowed) was produced when the 1020 bp fragment was digested was digested with *Taq* 1 (Fig. 3g, lane 3).

Figure 3g. *Taq* 1 digest of 1020 bp fragment of Atlantic DNA

Lane 1 and 4 = 100 bp marker
Lane 2 = undigested 1020 bp fragment
Lane 3 = *Taq* 1 digest of 1020 bp fragment (340 bp fragment indicated)

Development of PCR with primer sets CP113-5' 2\CP113-3' 2 and CP113-5' 1\CP113-3' 3

Effect of Mg and dNTP concentration

Mg concentration	2 and 3 mM
dNTP concentration	33 and 100 µM

DNA concentration	100 ng
Primer concentration	0.25 µM
Polymerase type and concentration	Ampli*Taq* 1 U
Buffer	10 mM Tris, 50 mM KCl (pH 8.3)

With primer set CP113-5' 2\CP113-3' 2 the expected 760 bp fragment (arrowed) was produced with both cultivars (Fig. 3h), with 2 mM Mg and 100 µM dNTP being the preferred concentrations (lanes 4 and 5).

With primer set CP113-5' 1\CP113-3' 3 the expected 700 bp (arrowed) was strongly produced with Maris Piper DNA (Fig. 3i, lanes 4, 6 and 8). The fragment was not strongly produced with DNA from Atlantic at the Mg and dNTP concentrations tested (lanes 3, 5, 7 and 9). With DNA from Atlantic a fragment corresponding to about 300 bp was produced (double arrow) with all Mg and dNTP concentrations tested. This was repeated three times and the result was consistently obtained.

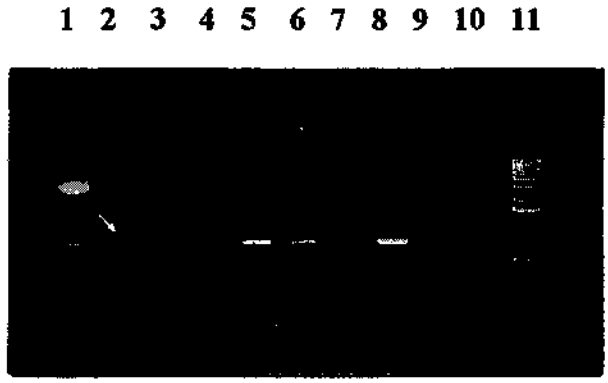

Figure 3h. Effect of $MgCl_2$ and dNTP concentration on amplification of Maris Piper and Atlantic DNA using primer set CP113-5' 2\CP113-3' 2

Lane 1 = 100 bp marker
Lanes 2 and 3 = 2 mM Mg\33 µM dNTPs (lane 2 = Maris Piper; lane 3 = Atlantic)
Lanes 4 and 5 = 2 mM Mg\100 µM dNTPs (lane 4 = Maris Piper; lane 5 = Atlantic)
Lanes 6 and 7 = 3 mM Mg\33 µM dNTPs (lane 6 = Maris Piper; lane 7 = Atlantic)
Lanes 8 and 9 = 3 mM Mg\100 µM dNTPs (lane 8 = Maris Piper; lane 9 = Atlantic)
Lane 10 = blank
Lane 11 = 1 kb marker

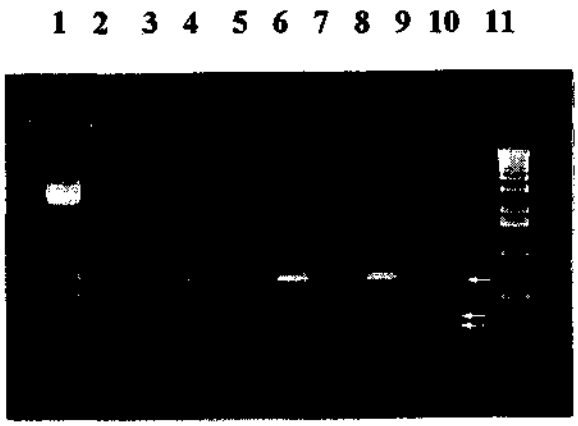

Figure 3i. Effect of $MgCl_2$ and dNTP concentration on amplification of Maris Piper and Atlantic DNA using primer set CP113-5' 1\CP113-3' 3

Lane 1 = 100 bp marker
Lanes 2 and 3 = 2 mM Mg\33 µM dNTPs (lane 2 = Maris Piper; lane 3 = Atlantic)
Lanes 4 and 5 = 2 mM Mg\100 µM dNTPs (lane 4 = Maris Piper; lane 5 = Atlantic)
Lanes 6 and 7 = 3 mM Mg\33 µM dNTPs (lane 6 = Maris Piper; lane 7 = Atlantic)
Lanes 8 and 9 = 3 mM Mg\100 µM dNTPs (lane 8 = Maris Piper; lane 9 = Atlantic)
Lane 10 = blank
Lane 11 = 1 kb marker

Appendix 4. Introduction to molecular markers, PCR and a glossary of terms

Introduction

The objectives of plant breeding are to improve the yield and the quality of a particular crop. To do this breeders use different cultivars and species to introduce genetic diversity into the crop through hybridisation. Depending on the crop and the characteristics being selected for, the time and effort to select for desirable plants vary. For crops such as fruit trees, it can take many years to breed new cultivars because of the long juvenile period. For other crops, such as tomato and potato it takes less time to develop new cultivars, as the time to assess for desirable characteristics is considerably reduced.

With potatoes, characteristics that can be readily observed, such as plant vigour or tuber size, shape and colour, are easy to select for. Other characteristics, such as yield and tuber starch composition, while relatively easy, require more effort as some laboratory analysis is required. Breeding for resistance to pests and diseases creates other problems, as it is necessary to challenge plants and select those that respond accordingly. Selection for resistance to powdery scab or potato cyst nematode, for example, requires that plants be grown in infected soil at a dedicated site.

Consequently plant breeding programmes are expensive to maintain and operate as they are labour intensive. Plant breeding is a numbers game and it is often necessary to screen many plants to find those with the most desirable combination of characteristics. Therefore approaches that result in the selection of plants with desirable features, as early as possible, should be particularly attractive to breeders.

For many years breeders have used a number of tricks to help speed up the selection process. One of the most widely used is linkage analysis. This involves using a marker that segregates with a characteristic of interest. Markers are usually for a morphological characteristic, such as leaf colour or a chemical such as an enzyme. Once such a marker has been associated or linked with a characteristic it can be used to monitor for the presence of the characteristic.

Molecular markers - what are they and why they are useful

The genetic analysis of plants has been recently improved by using molecular markers. In some ways molecular markers are similar to morphological and enzyme markers, except they are pieces of DNA associated with the presence or absence of a particular characteristic.

Two methods have commonly been used to obtain molecular markers: Restriction Fragment Length Polymorphisms (RFLPs) and techniques that have been developed from the Polymerase Chain Reaction (PCR).

Restriction Fragment Length Polymorphisms

The use of RFLP analysis in plant breeding depends on detecting natural variation in the sequence of DNA between individuals. These differences are found by cutting DNA into pieces (**fragments**) with **restriction** enzymes and separating them according to size (**length**) by using gel electrophoresis. Variability (**polymorphisms**) in the DNA is then looked for. This is done by transferring the DNA from the gel to a nylon membrane and incubating it with short pieces of DNA that are radioactive (probe), which can be seen on a X-ray film. This technique is known as Southern analysis.

By crossing plants with known characteristics and screening the parents and their offspring with RFLP analysis, it is possible to construct detailed genetic maps of a species. Preparing a genetic map with RFLP analysis is not a trivial task as it requires:

- many crosses to be done,
- different restriction enzymes to cut the DNA to be used,
- many different probes to be screened.

Once a genetic map of a species has been prepared and RFLP markers found for characteristics of interest, it is of immense value as it can help breeders:

- locate the general position of genes on a chromosome,
- to detect the presence of a gene in parents and their offspring.

Therefore RFLP analysis can improve the efficiency of breeding programmes by:

- better selection of parents,
- checking offspring while they are very young to see if a gene of interest is present.

Two important things to remember about RFLP markers are:

- they can be used to check for the presence of a characteristic well before it can be assessed by other means,
- a RFLP marker is only linked to a characteristic and doesn't necessarily provide any information about the gene.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a technique developed in the mid-1980s to multiply (amplify) a piece of DNA into billions of copies. The idea behind PCR is remarkably simple. DNA is made up of two strands which can be separated into single strands by heating. Short pieces of DNA (primers) are stuck to these single strands and a DNA **polymerase** (an enzyme that adds new DNA to a strand) is added. This makes new DNA starting from the primer and from two single strands, two double strands are made. This procedure of heating and adding more polymerase is repeated a number of times and each time the amount of DNA produced is doubled (the **chain reaction**). The procedure can be automated by using a special DNA polymerase that is stable during heating so that after 15 cycles, which takes about two hours, in excess of a billion copies can be made.

PCR has revolutionised many aspects of molecular biology and is extremely useful for:

- cloning genes,
- screening genetically engineered plants for the presence of newly introduced genes,
- obtaining DNA from extinct organisms,
- disease diagnosis,
- generating molecular markers.

PCR originally relied on having some knowledge of the sequence of gene of interest, as primers were specifically designed. If PCR is to be used for generating molecular markers there is an important but subtle difference. Instead of using 'designer primers', the primers are of arbitrary design. So instead of **amplifying** a specific piece of DNA, **random** pieces of DNA will be multiplied. When this DNA is run through a gel (like for RFLPs) bands of DNA of different sizes (**polymorphic**) can be seen. This development of PCR, to generate molecular markers is called **R**andomly **A**mplified **P**olymorphic **D**NA or RADPs. To use RAPDs to generate molecular markers, it is first necessary to have a population of plants with some knowledge of how they perform for the characteristic for which the markers are required. If say a marker was needed for resistance to late blight (*Phytophthora infestans*) how would you go about getting one? A plant known to be resistant is crossed with one susceptible and the offspring screened for their response to late blight. DNA is then extracted from the plants, but to speed up the procedure, the DNA from the offspring known to be resistant is pooled, as is the DNA from the susceptible offspring. Each of these two pooled samples is made up of individuals expressing late blight resistance or susceptible to late blight, but different for all other characteristics. These four groups of DNA (resistant parent, susceptible parent, resistant offspring and susceptible offspring) are analysed by PCR. By pooling the DNA samples, many different sets of primers can be tested at the same time. The aim is to find primers that produce a band of DNA on the gel that occurs only in the resistant offspring and resistant parent. This is the marker for

resistance to late blight. When a population of plants has to be screened to see how they would respond to late blight, the DNA is extracted and analysed by PCR using the correct primers and the appropriate band is looked for.

There are two things that need to be remembered about using RAPDs to generate molecular markers:

- to get the marker in the first place you need a population of plants that has been prepared specifically for the purpose,
- they are only useful to monitor for relatively simple characteristics that are controlled by one or a few genes, although this is rapidly changing.

In this project we have used three different approaches.

The initial approach utilised the RFLP marker CP113 that was found to be linked to the *H1* locus. To use the marker in a breeding programme it was necessary to use Southern analysis (as outlined earlier), which is technically demanding and rather tedious. By determining the genetic sequence of the CP113 marker it has been possible to design primers and convert the marker into a PCR based reaction. The developmental aspects are described in Appendix 3 and its application described in Sections 3, 4, 5 and 6.

The second approach used another marker (CD78) that had been found to be linked to the *H1* locus, but had not been sequenced. To use the CD78 marker it was necessary to utilise Southern analysis, the results of which are described in Sections 7.1 to 7.4.

The final approach evaluated the use of RAPDs, which has been slightly modified and assessed in Sections 7.5 to 7.8.

Glossary

allele	an alternative form of a genetic **locus**. A single allele for each locus is inherited separately from each parent
chromosome	the self replicating genetic structures of cells containing the cellular **DNA**, that bears in its nucleotide sequence, the linear array of genes
DNA	(deoxyribonucleic acid) the molecule that encodes genetic information. It is a double stranded molecule held together by weak bonds between the base pairs of nucleotides. The four nucleotides in DNA contain the bases: adenine (A), guanine (G), thymine (T) and cytosine (C). Base pairs only form between A and T and between C and G, thus the base pair sequence from each strand can be deduced from that of its partner
DNA polymerase	an enzyme which copies a **DNA** (or an **RNA**) molecule to produce a DNA copy
electrophoresis	a method of separating **DNA** or **RNA** molecules according to their size by 'sieving' them through an agarose gel. Agarose is a purified type of agar and is extracted from seaweed
ethidium bromide	a dye used to stain **DNA** or **RNA** that fluoresces when viewed with UV light
gene	an ordered sequence of nucleotides located in a particular position (**locus**) on a **chromosome** that encodes a specific protein
locus	(plural loci) the position on a **chromosome** of a **gene** or other chromosome marker
PCR	(Polymerase Chain Reaction) a method of amplifying **DNA** using a heat stable polymerase and (usually) two **primers**. Because the newly synthesised strands can be subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation and dissociation produce rapid and highly specific amplification of the desired sequence in a **DNA** template

primer	a short **DNA** sequence which 'primes' the addition of nucleotides to a DNA strand by **DNA polymerase**
polymorphism	difference in **DNA** sequence among individuals. Genetic variations occurring in more than 10% of a population would be considered useful polymorphisms for genetic linkage analysis
RAPD	(**R**andomly **A**mplified **P**olymorphic **D**NA) a type of PCR for generating **DNA** fingerprints that uses a single **primer** of arbitrary design. An individual primer will produce an identical DNA fingerprint (or profile) for the same cultivar. If the DNA is different (e.g. a different cultivar) a primer will produce a different DNA fingerprint, providing the **primer** detects a polymorphism
restriction enzyme	a protein that recognises specific short nucleotides and cuts DNA at those sites. Bacteria contain over 400 such enzymes that recognise and cut over 100 different DNA sequences
RFLP	(**R**estriction **F**ragment **L**ength **P**olymorphism) variation between individuals in DNA fragment sizes cut by specific **restriction enzymes**. Polymorphic sequences that result in RFLPs are used as markers on both physical maps and genetic linkage maps. RFLPs are usually caused by a mutation at a restriction enzyme cutting site
RNA	(ribonucleic acid) a nucleic acid found in the nucleus and cytoplasm of cells and plays a role in protein synthesis and other chemical activities. The structure of RNA is similar to DNA but uracil (U) replaces T