BIOCHEMICAL MARKERS FOR CULTIVAR IDENTIFICATION AND DISEASE RESISTANCE EVALUATION IN TOMATOES (*LYCOPERSICON ESCULENTUM*)

by

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ADH	alcohol dehydrogenase
APS	acid phosphatase
Aps-1	acid phosphatase-1 gene
Aps-1^{+}	acid phosphatase allele +
Aps-1^{1}	acid phosphatase allele 1
Aps-1^{3}	acid phosphatase allele 3
Aps-1^{4}	acid phosphatase allele 4
Aps-1	acid phosphatase-1 isozymes
Aps-2	acid phosphatase-2 isozymes
Aps-1^{+}	isozyme product of *Aps-1*$^{+}$ allele
Aps-1^{1}	isozyme product of *Aps-1*^{1} allele
Aps-1^{3}	isozyme product of *Aps-1*^{3} allele
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Cf-2	*Cladosporum fulvum* resistance gene on chromosome 6
Cf-9	*Cladosporum fulvum* resistance gene on chromosome 1
cM	centi Morgan
cv	cultivar
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetra acetic acid

EST	esterase
GOT	glutamate oxaloacetate transaminase
Got-2	glutamate oxaloacetate transaminase-2 isozyme
G-6-PDH	glucose-6-phosphate dehydrogenase
I1	*Fusarium oxysporum* resistance gene (race 1)
I2	*Fusarium oxysporum* resistance gene (race 2)
I3	*Fusarium oxysporum* resistance gene (race 3)
IEF	isoelectric focussing
L. peruvianum	*Lycopersicon peruvianum*
L. esculentum	*Lycopersicon esculentum*
M. arenaria	*Meloidogyne arenaria*
M. incognita	*Meloidogyne incognita*
M. hapla	*Meloidogyne hapla*
M. javanica	*Meloidogyne javanica*
Mi	Root knot nematode resistance gene
Mi-2	heat-stable root knot nematode resistance gene
MTT	methylthiazolyl tetrazolium
NIL	near isogenic line
PER	peroxidase
Per-2	peroxidase-2 isozyme
Pg3	stem rust resistance gene
6-PGDH	6-phosphogluconate dehydrogenase
PGI	phosphogluco isomerase
PGM	phosphogluco mutase

PMS	phenazine methosulphate
Pto	*Pseudomonase syringae* resistance gene
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphic DNA
Sm	*Stemphilium* resistance gene
spp.	species
TEMED	N,N,N',N'-tetramethylenediamine
TCA	trichloro-acetic acid
Tm-1	tobacco mosaic virus resistance gene (race 1)
Tm-2a	tobacco mosaic virus resistance gene (race 2)
Ve	*Verticillium* resistance gene
yv	yellow virescent gene

CHAPTER 1

LITERATURE REVIEW

1.1 Cultivar identification

A precise description of a newly bred cultivar is necessary to distinguish it from other cultivars of the same kind in order to protect the rights of plant breeders and producers (Arus, 1983; Bailey, 1983). A Certificate of Protection is granted for a novel cultivar, based on its distinctness from previously registered cultivars. Characteristics used to describe the novelty of a cultivar should be stable and observable throughout the protection period (Strachan, 1992). Traditionally, a new cultivar is distinct when it clearly differs by one or more identifiable morphological and physiological characteristics from all prior registered cultivars (Strachan, 1992). Cultivars of crops such as tomatoes and soybeans are morphologically similar which restrict their identification by morphological traits. Biochemical methods for cultivar identification are therefore required. Advantages of these methods are there greater sensitivity to detect genetic differences, the relative ease and shorter times needed to obtain results and low costs, since little or no greenhouse or land space is required. Despite these advantages, the application of biochemical methods in cultivar identification raises questions such as: Should differences in nonsense regions or non-coding regions of chromosomes be used? If procedures for some analyses are not standardised, will database comparisons be meaningful? What is a characteristic: a gene, an enzyme, a band, a base pair or another level of information? When such information is used to establish novelty, will it remain reproducible and stable during the protection period?

To answer some of these questions, proposals for the use of biochemical methods to identify cultivars were suggested by Bailey (1983). He emphasised that a DNA marker should not be overvalued as a blue print for cultivar identity as it could represent a non-coding area which has no effect on morphological, physiological or agronomic characteristics. Biochemical evidence used for cultivar identification also should be based on qualitative rather than quantitative differences. A qualitative difference is described as the presence or absence of a band in one cultivar compared to another. Quantitative differences are based on differences in band intensity which could be the result of inadequate extraction methods (Almgard and Clapham, 1975).

Bailey (1983) also accentuated the need for standardised conditions for cultivar identification methods to obtain reproducible results in any laboratory. For example, in the case of electrophoresis, extraction medium, gel and electrode buffer, gel porosity, constant voltage or current and staining methods are all conditions that must be standardised (Almgard and Clapham, 1975).

Environmental conditions should also be taken into consideration when standardising a technique for a specific crop (Bailey, 1983). Enviromental changes that could effect the stability of biochemical characteristics of a cultivar are temperature fluctuations (Werner and Sink, 1977), diseases, mechanical injuries (Shannon, 1986), air pollution (Curtis and Howell, 1971), the photoperiod to which the plant is exposed and nutritional status of the plant (Wilkenson and Beard, 1972). The type of tissue used and the age of the plant also influence gene expression (Marchylo *et al.*, 1980).

Biochemical methods typically used for cultivar identification are protein and isozyme analysis which detect differences at the protein level and restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) that detect differences at the DNA level.

Proteins can be separated by charge and size in a polyacrylamide gel (PAGE), using a homogenous or gradient gel, whereas isoelectric focusing will separate proteins based on differences in iso-electric points. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) separates denatured proteins according to size (Weber and Osborn, 1969).

No literature exists on the identification of tomato cultivars by means of unique protein banding patterns. However, cultivars of another *Solanaceae* species, namely potatoes, were identified by protein banding patterns after SDS-PAGE, gradient-PAGE, native-PAGE and IEF (Stegemann, 1983). Shewry *et al.* (1978) used SDS-PAGE to fractionate the proteins extracted from wheat grains to discriminate between cultivars. Other crops, eg. barley (Shewry *et al.*, 1978), maize (Stegemann, 1979), rice (Padhye and Salunkhe, 1979), peas (Cooke, 1984) and soybeans (Blogg and Imrie, 1982) were identified by protein profiles obtained after native-PAGE or SDS-PAGE.

Much of the appeal to use isozymes in cultivar identification is their apparent environmental stability, extended polymorphism and the relatively cheap and easy separating techniques (Bailey, 1983). Isozymes are multiple forms of the same enzyme and have similar, but not necessarily identical properties. For example, isozymes can catalyse the same reaction, but can differ in their kinetics. Isozymes are formed in three ways (Harris and Hopkinson, 1976),

namely (a) multiple gene loci coding for similar, though structurally distinct, polypeptide chains of the enzyme. This is probably the result of gene duplication which occurred during evolution or the subsequent divergence in structure as a result of point mutations. (b) Multiple allelism at a single locus with each allele coding for structurally distinct versions of a particular polypeptide chain. In other words, at any given locus a number of different alleles may occur in a population of individuals with the result that the primary structures of the isozymes will differ from one individual to another. Multiple allelism and multiple loci which result in isozyme formation may be distinguished by the fact that the former results in differences in isozyme patterns between individuals of a species, whereas the latter are in general common to all members of a species or cultivar. (c) Thirdly, isozymes can be formed due to post-translational modifications of the enzyme structure. As multiple loci and allelism provide the basis of the primary amino acid sequence of the polypeptide chains, post-translational changes account for secondary modifications of the enzyme structure. Possible structural modifications that may occur involve deamidation of glutamine or asparagine residues on the surface of the molecule, addition of phosphate groups, addition or removal of carbohydrate groups, proteolytic cleavage and aggregation or polymerisation.

Isozymes which differ significantly in amino acid composition will have dissimilar net ionic charges, molecular sizes and shapes. In the presence of an electric field and a semiporous gel medium, these differences cause iso-forms of an enzyme to migrate at different rates and thus, different banding patterns after enzyme specific staining. Depending on the enzyme under investigation, an appropriate staining procedure is employed to reveal the relative positions of each isozyme band. These stained bands together constitute the zymogram.

Although the plant's growing environment has no effect on the amino acid sequence of a particular isozyme, the environment can effect gene activity and determine if, as well as when and how much of a particular isozyme is produced in the organ or tissue chosen for extraction (Werner and Sink, 1977).

Although to date isozymes have not been used for the identification of tomato cultivars, they have been used for several other objectives. For example, the polymorphism in alcohol dehydrogenase (ADH) was used for testing the genetic purity of F1 hybrids (Tanksley and Jones, 1981; Van den Berg, 1991). Parentage determination of 35 offspring combinations of grape cultivars was also achieved by the use of two isozyme systems (Ohmi *et al.*, 1993). Isozymes, genetically linked to economical and agricultural important genes, such as disease resistance genes, are also used as markers to detect disease resistances in breeding lines selected in a breeding program. This topic is fully discussed in section 1.2.

Isozymes have been widely used for cultivar identification of other crops. For example, garlic cultivars (Pooler and Simon, 1993), wild barley (Zhang *et al.*, 1993), macadamia (Vithanage and Winks, 1992), lettuce (Cole *et al.*, 1991), red maple (Tobolski and Kemery, 1992), walnuts (Aleta *et al.*, 1990) and olive cultivars (Trujillo *et al.*, 1990) have been identified by distinct isozyme patterns.

In circumstances where isozymes may lack adequate polymorphism to distinguish between closely related cultivars such as tomatoes, DNA based methods such as RFLP's and/or RAPD's may be used. Theoretically, these methods can screen the DNA of the whole genome, in comparison to protein based methods, which increase the probability to detect

genetic differences between cultivars.

Restriction fragment length polymorphism (RFLP) is based on the digestion of total genomic DNA with restriction enzymes. The digested DNA is separated on an agarose gel and blotted (Southern blot) onto a nylon membrane as single stranded DNA. After hybridization with a labelled probe (single stranded DNA or RNA), bands are visualized on e.g. X-ray film (Fig. 1.1, Bernatzky and Tanksley, 1989). Probes for the identification of RFLP's are usually obtained from cDNA or genomic clones.

RFLP's usually produce more polymorphism than isozymes, since it is not restricted to structural genes. The whole plant genome can be screened by cDNA and genomic clones (Bernatsky and Tanksley, 1986). Despite this advantage, it is not a very popular method for cultivar identification as costs involved are high and it is a more laborious and complex method to perform than isozyme analysis (Beckmann and Soller, 1986).

Only two publications on identification of tomato cultivars by RFLP's have been reported. Helentjaris *et al.* (1985) tested a number of tomato cDNA clones and found one clone which could detect differences in banding patterns between two cultivars which are genetically not closely related. Broun *et al.* (1992) used a telomeric and a TGRI probe as RFLP markers to distinguish between closely related tomato cultivars. A mutation rate of 2 % per generation occurs in the parts of the chromosomes where these probes hybridise which unfortunately diminishes their effectiveness.

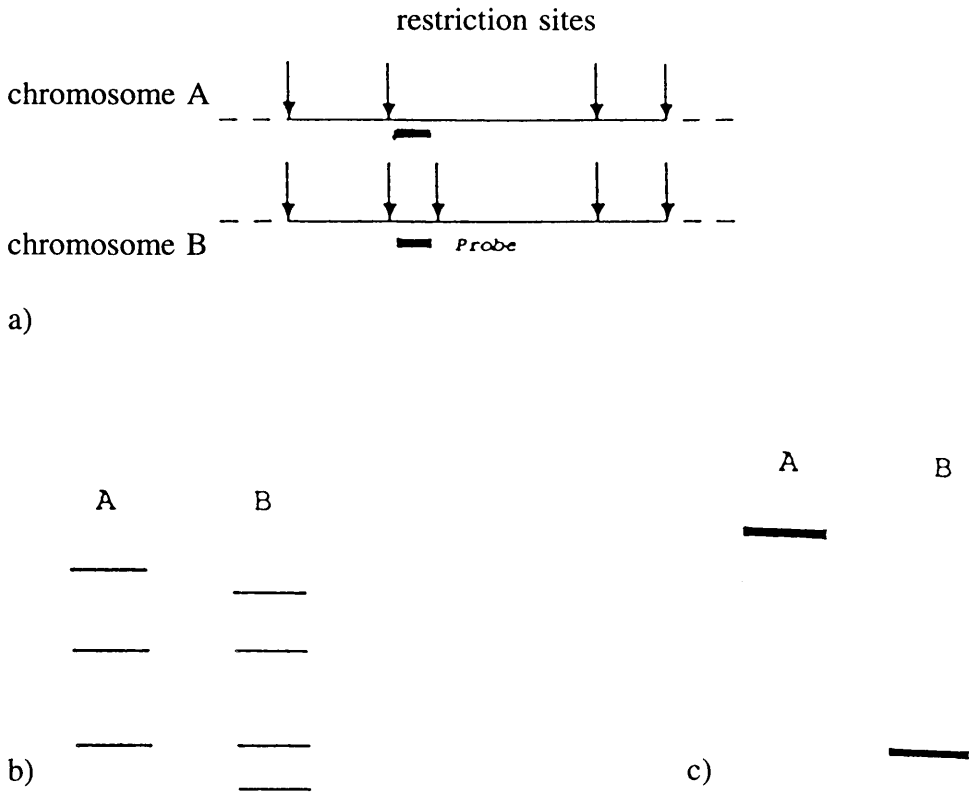


Figure. 1.1. A schematic representation of the detection of polymorphism by RFLP's. a) Chromosomes A and B differ in the position of a restriction site (arrows). b) These two DNA's are digested with the restriction enzyme and separated electrophoretically and transferred to a membrane. c) a DNA fragment on the membrane, which is homologous to the radioactively labelled probe, is hybridised in solution. This is then placed against a X-ray film to be visualised.

RFLP's of tomatoes were also extensively used in the construction of a high-density-linkage-map. Approximately 1000 RFLP markers with an average distance of 1.2 cM between them were mapped on all the tomato chromosomes (2n = 24) (Tanksley, Personal communication). Some of these RFLP markers are genetically linked to economical important genes such as disease resistance genes, and could therefore be used to trace these characteristics in a breeding program or to evaluate cultivars for disease resistance (see section 1.2).

However, RFLP markers have been successfully used as a tool for cultivar identification of other crops. Parent and Page (1992) and Nybom and Hall (1991) identified raspberry cultivars by non-radioactive DNA fingerprinting. Blueberry cultivars (Haghighi and Hancock, 1992), avocado (Lavi *et al.*, 1991), peanut (Kochert *et al.*, 1991), carnation (Vainstein *et al.*, 1991) apples (Nybom *et al.*, 1990; Nybom, 1990), barley (Graner *et al.*, 1990) and soybean cultivars (Keim *et al.*, 1989; Apuya *et al.*, 1988) were all identified with the aid of RFLP markers.

Recently, a new class of genetic markers namely, random amplified polymorphic DNA (RAPD's) were investigated as a tool for the identification of cultivars. These markers share most of the advantages of RFLP markers (Boltstein *et al.*, 1980) without many of their disadvantages (Williams *et al.*, 1990). The whole genome can be screened with this method and it is quicker, less complex and less laborious to perform than RFLP's. Costs involved are lower (Ragot and Hoisington, 1993), very small amounts of template DNA is required, knowledge of gene sequences is not a prerequisite and no radioactivity is needed (Deragon and Landry, 1992).

The RAPD method is based on the polymerase chain reaction (PCR) protocol with two important differences, namely the RAPD method uses only single and random oligonucleotide primers of which the priming site targets are unknown (Welsh and McClelland, 1990). The RAPD method is performed by adding a single 10-mer oligonucleotide of a random DNA sequence (comprising between 50 - 70% G/C) to genomic DNA in the presence of a thermostable DNA polymerase and a suitable buffer. It is then subjected to temperature cycling conditions typical of the PCR reaction. The products of the reaction depend on the sequence and length of the oligonucleotide (the primer), as well as the reaction conditions. At an appropriate annealing temperature during the thermal cycle, the single primer binds randomly to sites on opposite strands of denatured genomic DNA that are within 5000 bp from each other (Williams, *et al.*, 1991a). A mixture of amplified products are produced, separated by agarose or polyacrylamide gel electrophoresis (Harada *et al.*, 1993) and visualised by ethidium bromide. The whole plant genome can be screened with a number of random primers. Theoretically, the banding patterns of one individual should differ from that of another which is genetically distinct. A DNA fragment that is polymorphic in one individual is a potential genetic marker which can be used for cultivar identification (Rafalski *et al.*, 1991), disease resistance evaluation, the construction of a genetic linkage map (Lashari and Saul, Personal communication; Weining and Langridge, 1991), parentage determination (Harada *et al.*, 1993; Heun and Helentjaris, 1993; Welsh *et al.*, 1991) and also F1 hybrid purity testing (Livneh *et al.*, 1992).

A few modifications to the standard RAPD procedure were suggested by Williams *et al.* (1991a) to increase the probability of detecting polymorphism3 between closely related species, namely:

(a) Individual RAPD bands may be excised from the gel and re-amplified for use as hybridization probes (Williams *et al.*, 1991b). (b) Gels containing RAPD bands can be blotted onto a permanent membrane and hybridized with the probes derived from selected RAPD bands. (c) Digesting genomic DNA with frequently-cutting restriction enzymes prior to the amplification reaction is another way of increasing the probability of polymorphism. (d) Primers can be used in pairs to vary the number and type of RAPD bands. (e) RAPD gels stained with silver showed more bands than gels stained with ethidium bromide (Bassam *et al.*, 1991).

Although RAPD's have been introduced only recently, they are already widely used as genetic markers for many crops. Cultivar identification by means of RAPD markers were implemented for crops like onions (Wilkie *et al.*, 1993), roses (Torres *et al.*, 1993), papaya (Stiles *et al.*, 1993), apples (Koller *et al.*, 1993) and wheat (Devos and Gale, 1992) to name a few. The only report that included some aspects on cultivar identification of tomatoes was published by Vosman *et al.* (1992). They found that two of their primers could distinguish between two cultivars that are not closely related, but modern cultivars that are genetically more similar could not be distinguished by the same two primers. RAPD's of tomatoes were also linked to economical important genes, such as disease resistance. This aspect is discussed in section 1.2.

In less than four years time, this novel technique has become widely accepted as a simple, rapid, safe and inexpensive method for identifying polymorphism at the level of the genome. With the commercial availability of hundreds of primers at low cost, the generation of numerous genetic markers in large varieties of species is now expected to proceed at an

unprecedented rate.

1.2 Evaluation of disease resistance

Traditionally, the breeding of new tomato cultivars is based on the selection of desirable breeding lines by examining phenotypic characteristics such as disease resistance and fruit quality. For example, a disease resistant gene is incorporated into a sensitive plant by a crossing between the sensitive plant (with appropriate characteristics) and a tolerant one. The progeny of the crossing are then inoculated with a pathogen or infested with, for example, nematodes. After a few weeks the plants are visually evaluated for symptoms caused by the pathogen. This process requires greenhouse or field space which makes selection of breeding lines time consuming, expensive and labour intensive. Environmental factors such as temperature also has an effect on the results and an expert is needed to identify the symptoms caused by the pathogen. Plants also cannot be screened for more than one pathogen at a time and "new" pathogens are kept under quarantine with the consequence that breeding lines cannot be evaluated for resistance to the latter pathogens. A period of ten years could easily pass from the initial crossing until a new and improved cultivar is released. The phenotype of a plant is not only influenced by its genes but is also dependent on environmental factors. Therefore, undesirable environmental conditions can disguise desirable genotypes of a plant which may lead to the selection of plants with unacceptable genetic potential (Gresshoff, 1992).

A modern trend in plant breeding is to combine traditional breeding methods with molecular methods performed in a laboratory. The evaluation of disease resistance by molecular markers

has advantages that makes this kind of evaluation very attractive to plant breeders. Evaluation of breeding lines in a laboratory is much quicker, less labour intensive, cost effective and environmental factors have no influence on the results. More than one disease resistant gene can be detected at a time on the same plant without exposure to pathogens which still may be under quarantine.

Some isozymes, RFLP's and RAPD's are already used as markers to evaluate tomato breeding lines and cultivars for disease resistance in breeding programs. Tomato isozymes of acid phosphatase (APS), peroxidase (PER) and glutamate oxaloacetate transaminase (GOT) enzymes were found to be genetically linked, by chance, to some economical important genes. The isozyme Got-2 is genetically linked to the *Fusarium oxysporum* race 3 resistance gene (Bournival *et al.*, 1989) and the male-sterile gene could be transferred into breeding lines by selection of the Per-2 marker (Tanksley and Zamir, 1988). *Aps-1* , which encodes acid phosphatase-1 isozymes, is closely linked to *Mi*, a gene conferring resistance against nematodes (Medina-Filho, 1980).

RFLP markers have a practical implication for a breeding programme if there is a close linkage between marker and an economical important trait (Burr *et al.*, 1983). A comparison between the high-density-linkage-map and a morphological map of tomatoes allows identification of morphological traits linked to RFLP markers. Table 1.1 shows disease resistant genes which are genetically linked to RFLP markers in tomatoes. These RFLP markers can be radioactively labelled and used in the Southern blot technique to identify individual plants with the desired characteristic.

Table 1.1. Disease resistant genes of known phenotype that have been mapped onto the molecular map of tomato by means of RFLP markers. References are for mapping of loci onto a molecular linkage map and/or source of RFLP marker used for such mapping.

GENE	PHENOTYPE[1]	CHROMOSOME	REFERENCE
Cf-2	*Cladosporum fulvum*	6	Jones *et al.*, 1991
Cf-9	*Cladosporum fulvum*	1	Jones *et al.*, 1991
I1	*Fusarium oxysporum* race 1	7	Sarfatti *et al.*, 1989
I2	*Fusarium oxysporum* race 2	11	Sarfatti *et al.*, 1989
I3	*Fusarium oxysporum* race 3	7	Bournival *et al.*, 1989; Tanksley and Costello, 1991
Mi	Root knot nematodes	6	Messegeur *et al.*, 1991
Pto	*Pseudomonas syringae*	5	Martin *et al.*, 1991
Sm	*Stemphilium*	11	Behare *et al.*, 1991
Tm-1	tobacco mosaic virus	2	Levesque *et al.*, 1990
Tm-2a	tobacco mosaic virus	9	Young *et al.*, 1988
Ve	*Verticillium*	7	Juvick *et al.*, 1991

[1]Resistance to the listed pathogens

Although RFLP markers are available for a number of important disease resistant genes for tomatoes, the application of these probes on a regular basis for evaluating breeding lines in a breeding programme has some drawbacks. If RFLP's are used on a routine basis, a specialised laboratory must be established which is expensive. The procedure is also relatively complex to perform and a well trained technician is needed to perform the procedures. Radioactivity is often used in the protocol which is hazardous and requires specialized procedures and equipment.

With the development of the RAPD procedure (Williams *et al.*, 1990; Welsh and McClelland, 1990), a less complex and cost effective method with the same capabilities as RFLP's was introduced. A number of reports have already been published on the use of RAPD's to identify molecular markers for evaluating breeding lines for disease resistances. Martin *et al.* (1991) used the RAPD procedure in conjunction with near-isogenic lines (two lines which differ only with respect to a single gene) to identify sequences linked to the gene of interest. They used a pair of tomato near-isogenic lines that differ for a region on chromosome 5 that contains a gene (*Pto*) conferring resistance to *Pseudomonas syringae*. One hundred and forty four random primers were used to screen these lines and seven amplified DNA fragments were identified that were present in one but not the other line. Four of these seven fragments were further investigated and three were confirmed by segregation analysis to be tightly linked to the *Pto* gene. Linked DNA fragments identified by this method are useful for detecting the presence of the target gene in plant breeding and if tightly linked, as starting material for a chromosome walk to isolate the gene (Tanksley *et al.*, 1989).

Klein-Lankhorst *et al.* (1991b) used single and combined RAPD primers to identify DNA

fragments linked to the *Mi* gene (nematode resistance) in tomatoes. They also used near-isogenic lines (NIL's) which only differ with respect to the *Mi* gene on chromosome 6.

The same strategy was followed by Penner *et al.* (1993) on oats where they screened near-isogenic lines for polymorphic DNA fragments linked to the stem rust resistance gene *Pg3*. Two primers were identified which amplified DNA fragments that were polymorphic for the lines analyzed. One RAPD fragment was shown to be linked to the *Pg3* locus, while the other one was not linked at all. Paran *et al.*, (1991) used lettuce NIL's to identify RAPD markers linked to powdery mildew resistance.

When near-isogenic lines of a crop are not available the "bulk segregant analysis" method as developed by Michelmore *et al.* (1991) could be used as an alternative method to find RAPD markers linked to a specific gene. This method is illustrated in Figure 1.2. Bulked segregant analysis involves the RAPD screening of two parents (P1 and P2 in Fig. 1.2 a), one containing the dominant resistant gene (RR) and the other parent the recessive sensitive rr gene (Fig. 1.2 a). The two parents are also genetically different by other genes and therefore not near-isogenic lines. The F1 progeny of these parents are screened with the same primers and it is expected that each F1 plant will exhibit the same pattern (Fig. 1.2 b). The F2 progeny are then screened in a greenhouse for susceptibility (rr) and tolerance (RR or Rr) by traditional methods. Each pool, or bulk of plants (RR/Rr and rr plants), contains individuals selected to have identical genotypes for a particular genomic region (target region or resistance locus) but random genotypes at loci unlinked to the selected region (Fig. 1.2 d).

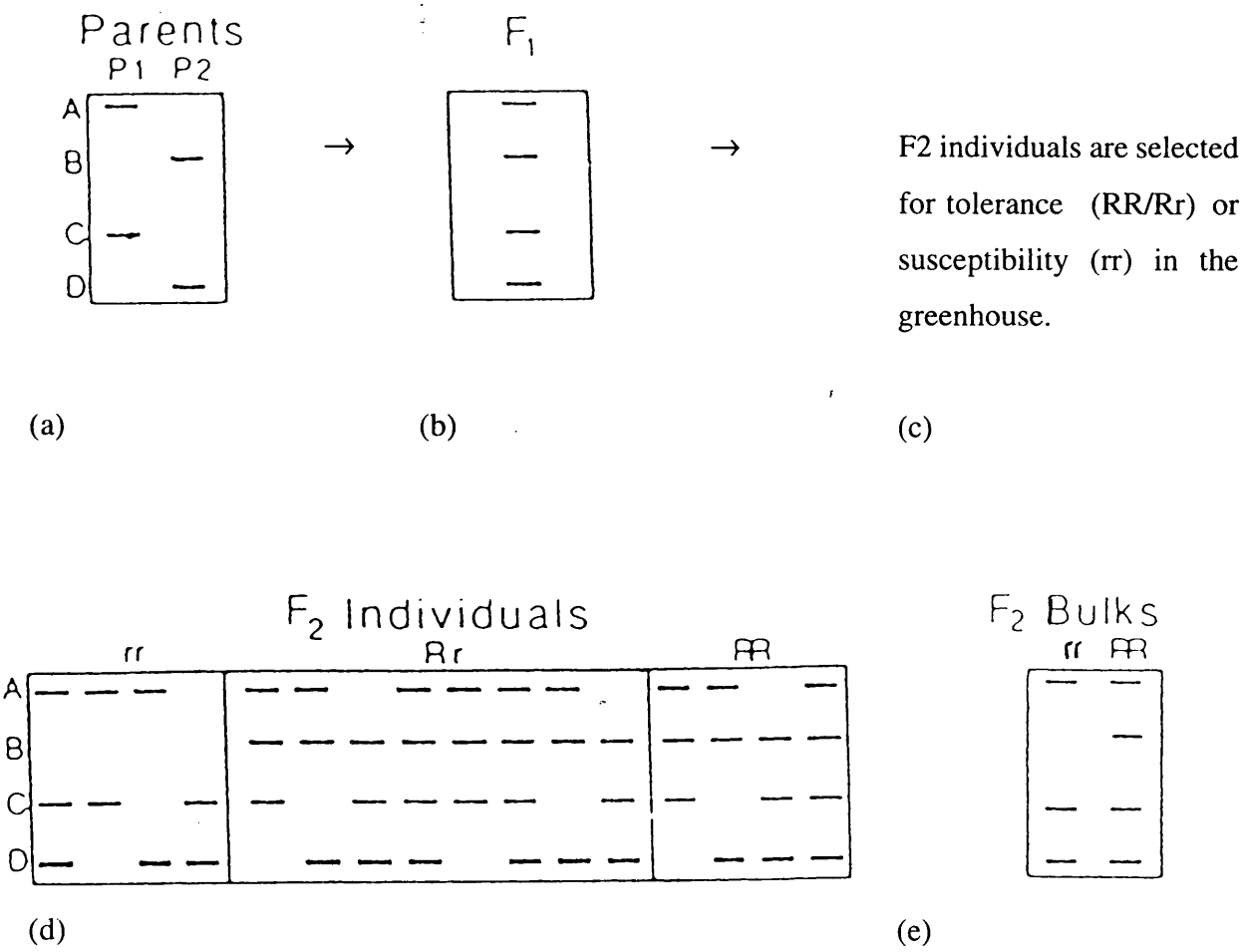


Figure 1.2. **Bulk segregant analysis** (a) The schematic shows four RAPD bands of loci A - D detected by a single primer in two parents (P1 and P2). (b) RAPD bands of their F1 progeny. (c) The F2 individuals are evaluated for tolerance (RR/Rr) or susceptibility (rr) with traditional methods and (d) shows the RAPD bands of these F2 individuals. (e) The bulks (RR/Rr and rr) are pooled and analysed with the same primer. The dominant allele at locus B is linked to the R allele and therefore is polymorphic between the two F2 bulks. Loci A, C and D are polymorphic between the parents, but unlinked to the resistance locus and therefore appear non-polymorphic between the bulks.

DNA from the two bulks are then screened for differences with the same RAPD primers used previously. Linkage between a polymorphic marker and the target locus is confirmed and qualified by using the segregating population (F2) from which the bulks were generated. Bulk segregant analysis allows the rapid screening of many loci and therefore the identification of linked markers in the target region.

1.3 Aims of the study

The aims of the study were to investigate methods for biochemical identification of genetically similar tomato cultivars which can be used in combination with morphological and physiological characteristics to obtain Plant Breeders Rights on novel cultivars. Such a method need to be implementable on a routine basis and therefore should be relatively easy to perform and reproducible between laboratories. Secondly, the genetic linkage between the acid phosphatase-1 isozyme (Aps-1^1) and the *Mi* gene (gene for resistance to root knot nematode) were investigated for implementation of linkage analyses in breeding programmes.

CHAPTER 2.

PROTEINS AS MARKERS FOR CULTIVAR IDENTIFICATION

2.1 Introduction

Protein separation in gels was initiated as early as 1955 (Smithies, 1955). Using starch gels, the first species identifications were undertaken for fish (Thompson, 1960) followed by cultivar identification of cereals (Elton and Ewart, 1962).

Polyacrylamide gels were introduced during the late 1950's by Raymond and Weintraub (1959) and the first PAGE (polyacrylamide gel electrophoresis) protein patterns of cultivars were published three years later (Stegemann and Loeschcke, 1962). Fox *et al.* (1963) used PAGE in tubes for separating proteins from legume cultivars and the number of papers on this subject have since increased exponentially, illustrating the usefulness of protein patterns for discriminating between cultivars.

Proteins as markers for identification of tomato cultivars have never been reported, possibly due to insufficient variation between protein profiles of cultivars. However, SDS-PAGE and isoelectric focusing (IEF) of soluble proteins of the mature floral organs of tomatoes (the sepals, petals, stamen and gynoecium) showed differences in protein patterns (Sawhney *et al.*, 1985). Protein determinations of the various floral organs showed that stamens contained the greatest amount and the petals the least amount of protein. SDS-PAGE showed that each organ-type contained many polypeptide bands, some of which were common to all organs

while others were present in some but not all organs, and still others were specific to an organ type.

Protein analyses for other crops was used as guideline for the planning of a similar technique to discriminate between tomato cultivars. Potatoes, which belong to the same family as tomatoes, *Solanaceae*, showed variation in protein patterns between different cultivars. Potato proteins were separated by means of native polyacryamide gel electrophoresis (native-PAGE, 6%), gradient-PAGE (5 to 25%), SDS-PAGE (6%) and isoelectric focusing (pH gradient between 4 and 9) (Stegemann, 1983). Native-PAGE was found to be the most appropriate method to distinguish between potato cultivars (Stegemann, 1983). Both leaves (Daoyu and Lawes, 1990; Hart and Bhatia, 1967) and seeds (Aiken and Gardiner, 1990; Marcylo, 1987; Shah and Stegemann, 1983; Blogg and Imrie, 1982; Marcylo and LaBerge, 1980) of a number of crops were used in these studies.

The present study was conducted to determine if protein separating techniques such as gradient-PAGE, IEF and SDS-PAGE have sufficient resolving power to discriminate between tomato cultivars. Proteins of both seeds and leaves were tested.

2.2 Materials and Methods

2.2.1 Plant material

Cultivars chosen for identification form part of the tomato breeding programme at Roodeplaat, Pretoria. These cultivars and their country of origin are listed in Table 2.1.

Table 2.1. Cultivars used for analysis.

CULTIVAR	COUNTRY OF ORIGIN
1. Rotam-4	South Africa
2. Rotam-3	South Africa
3. Rodade	South Africa
4. Flora-Dade	Florida, USA
5. Stevens	South Africa
6. NC EBR-1	North Carolina, USA
7. NC EBR-2	North Carolina, USA
8. Karino	South Africa
9. Ld2048	South Africa
10. Irat L3	France
11. Walter	Florida, USA
12. L1051	South Africa
13. Corde'oc	France
14. Dawie[1]	Israel
15. M88/44[2]	South Africa
16. M88/68[2]	South Africa

[1] Code name for cultivar
[2] Advanced breeding lines (F10)

2.2.2 Protein extraction

Tomato plants listed in Table 2.1 were grown in a greenhouse with constant humidity, temperature and photoperiod in pots of 20 cm in diameter. The same amount of water and nutrients were applied under disease and pest free conditions.

Crude extracts of water soluble proteins were obtained from 0.02 g of mature (full grown) tomato leaves and separated by IEF and gradient-PAGE. The leaves were crushed in liquid

nitrogen with a mortar and pestle, whereafter 200 µl of extraction buffer (10 ml of 0.25 M Tris-HCl pH 6.8, 4 ml 10 % glycerol, 2 ml ß-mercaptoethanol and 16 ml of distilled water; Chen and Luthe, 1987) was added. The extractions were done at 4°C and extracts centrifuged at 10 000 x g for 5 minutes. The entire supernatant was loaded onto the gel.

Protein extracts from mature leaves (0.02 g) as well as dormant seeds (0.03 g) were also separated by SDS-PAGE. Extracts for SDS-PAGE were treated similarly as above, except that the extraction buffer contained 2 % SDS and that 5 µl of the seed extracts were loaded onto the gel.

Protein concentrations were determined by the Standard Assay Procedure Bio Rad (Bio Rad, USA) kit. Protein standards of 0.2 to 1.4 mg/ml were prepared with bovine serum albumin (BSA; Sigma). Five millilitres of a 1:4 diluted Coomassie blue stain (as supplied in the Standard Assay Procedure Bio Rad kit) was added to 0.1 ml of standard and sample. Absorbances were measured at 595 nm after five minutes.

2.2.3 Electrophoresis

Vertical isoelectric focusing was used to separate leaf proteins. Gels (6 %) were prepared by mixing 2 ml of 30 % (w/v):1.0 % (w/v) acrylamide:N,N'-methylene bisacrylamide; 7 ml of distilled water; 2.4 ml of 50 % glycerol and 0.6 ml of ampholytes (Sigma; pH-range of 3 - 10). The mixture was degassed before the addition of 50 µl of 10 % (w/v) ammonium persulphate and 20 µl of N,N,N',N'-tetramethylene diamine (TEMED) (Robertson *et al.*, 1987). A mini gel electrophoresis apparatus (BioRad) of a 80 x 100 x 1.5 mm gel format was

used. Gels were left overnight at room temperature to polymerize. Samples were mixed in a 1:1 ratio with 60 % glycerol and 4 % ampholytes before loading onto the gel.

The cathode solution consisted of 0.025 M sodium hydroxide and the anode solution of 0.02 M acetic acid, prechilled to 4 °C. Electrophoresis was performed at room temperature (25°C) for 1.5 hours at 200 constant volt, whereafter it was increased to 400 constant volt for an additional 1.5 hours. After electrophoresis was completed, gels were rinsed with distilled water and the pH-gradient was measured with a flat bottom pH electrode (Beckman) on the gel surface.

After electrophoresis was completed, gels were washed in 10 % trichloroacetic acid (TCA) for 10 minutes and then submerged into 1 % TCA for two hours to remove the ampholytes. Gels were rinsed with water and stained with 0.25 % Coomassie blue in 45 % methanol and 10 % acetic acid for 10 minutes and rinsed with water before they were destained in 45 % methanol and 10 % acetic acid over activated charcoal (Robertson *et al.*, 1987).

Gradient-PAGE was also used to separate leaf proteins. The procedure is based on a method described by Suurs *et al.* (1989). A peristaltic pump and magnetic stirrer were used to cast a 5 - 20 % gradient polyacrylamide gel. The 5 % gel solution consisted of 7.5 ml of 40 % (w/v):1.6 % (w/v) polyacrylamide:N,N'-methylene bisacrylamide; 15 ml of 1.5 M Tris-HCl, pH 8.9 containing 0.24 % TEMED; 31.5 ml of distilled water and 6 ml of 0.2 % ammonium persulphate was added just before casting. The 20 % gel solution consisted of 30 ml of 40 % (w/v):1.6 % (w/v) polyacrylamide:N,N'-methylene bisacrylamide; 15 ml of 1.5 M Tris-HCl, pH 8.9 containing 0.24 % TEMED; 9 ml of distilled water; 6 g sucrose, 1 mg of

bromophenolblue and 6 ml of 0.2 % ammonium persulphate. The gel was left overnight to polymerise and just before use, a 2.6 % polyacrylamide stacking gel was poured on top of the gradient gel. It consisted of 4 ml of 40 % (w/v):1.6 % (w/v) polyacrylamide:N,N'-methylene bisacrylamide; 2.5 ml of 0.32 M Tris-HCl, pH 8.6; 36 µl of TEMED; 24.5 ml of distilled water and 6 ml of 0.2 % ammonium persulphate. Electrophoresis was performed at room temperature (25 °C) for 18-20 hours (Leguay and Jouanneau, 1987) at constant current (15 mA) in electrode buffer containing 14.4 g glycine ; 66 ml of 0.75 M Tris-HCl (pH 8.8) and 34 ml of distilled water. A Bio Rad electrophoresis apparatus with a gel format of 18 x 20 cm x 1.5 mm was used.

Gels were stained according to the method of Juge-Allbrey *et al.* (1983) for 4 - 6 hours. The stain consisted of 50 % methanol, 10 % acetic acid and 0.25 % (w/v) Coomassie blue R250. Gels were destained in methanol and acetic acid (5:1).

SDS-PAGE was used for the separation of leaf as well as seed polypeptides. The separating gel (15 %) consisted of 17 ml of 30 % (w/v):0.8 % (w/v) polyacrylamide:N,N'-methylene bisacrylamide; 17 ml of 0.75 M Tris-HCl pH 8.8 and 0.4 ml of 10 % sodium dodecyl sulphate (SDS). One millilitre of 10 % ammonium persulphate and 12 µl of TEMED were used to polymerize the gel. The stacking gel (6.3 %) contained 3 ml of 30 % (w/v):0.8 % (w/v) polyacrylamide:N,N'-methylene bisacrylamide; 10 ml of 0.25 M Tris-HCl, pH 6.8 and 0.2 ml of 10 % SDS. The electrode buffer consisted of 14.4 g glycine; 66 ml of 0.75 M Tris-HCl (pH 8.8); 0.1 g SDS and 34 ml of distilled water (Laemmli, 1970). Electrophoresis was performed at room temperature (25 °C) for 18-20 hours (Leguay and Jouanneau, 1987) at constant current (15 mA). A Bio Rad electrophoresis apparatus with a gel format of 18 x 20

cm x 1.5 mm was used.

Gels were stained according to the method of Juge-Allbrey *et al.*, (1983) for 4 - 6 hours.

2.3 Results

2.3.1 Protein concentrations

Table 2.2 shows the protein concentrations of leaves (0.02 g) and of seeds (0.03 g). Similar protein concentrations were extracted from different cultivars for seeds as well as for leaves.

Table 2.2. Protein concentrations of tomato leaf and seed extracts.

CULTIVAR	Protein in supernatant (mg/ml)	
	LEAVES	SEEDS
Rotam-3	6.4	29.0
Rotam-4	6.2	29.4
Rodade	6.0	30.4
Flora-Dade	6.4	29.4
Stevens	4.8	30.4

2.3.2 Protein profiles

Figures 2.1, 2.2, and 2.3 show protein patterns of tomato leaf extracts separated by isoelectric focusing (pH 3 - 10), gradient-PAGE (5 - 20 %) and SDS-PAGE (15 %), respectively. Cultivars listed in Table 2.1 are represented by lanes 1 - 16. No differences in protein patterns between cultivars were noticed after IEF or gradient-PAGE.

No qualitative differences in patterns were detected after SDS-PAGE (Figure 2.3), but quantitative differences are noticed in e.g. lanes 5 and 9. A possible explanation for this inconsistency could be the dissimilar loading of supernatant onto the gel, since this variation was not always observed in subsequent experiments.

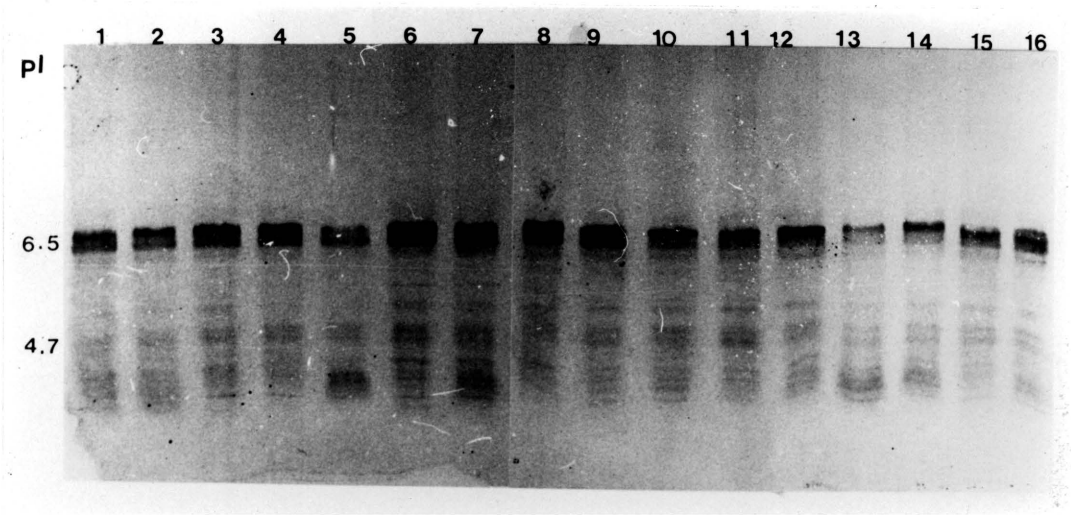


Figure 2.1. Protein profiles of tomato leaves separated by IEF with a pH gradient of 3 - 10. Lanes 1 - 16 show different cultivars (see Table 2.1)

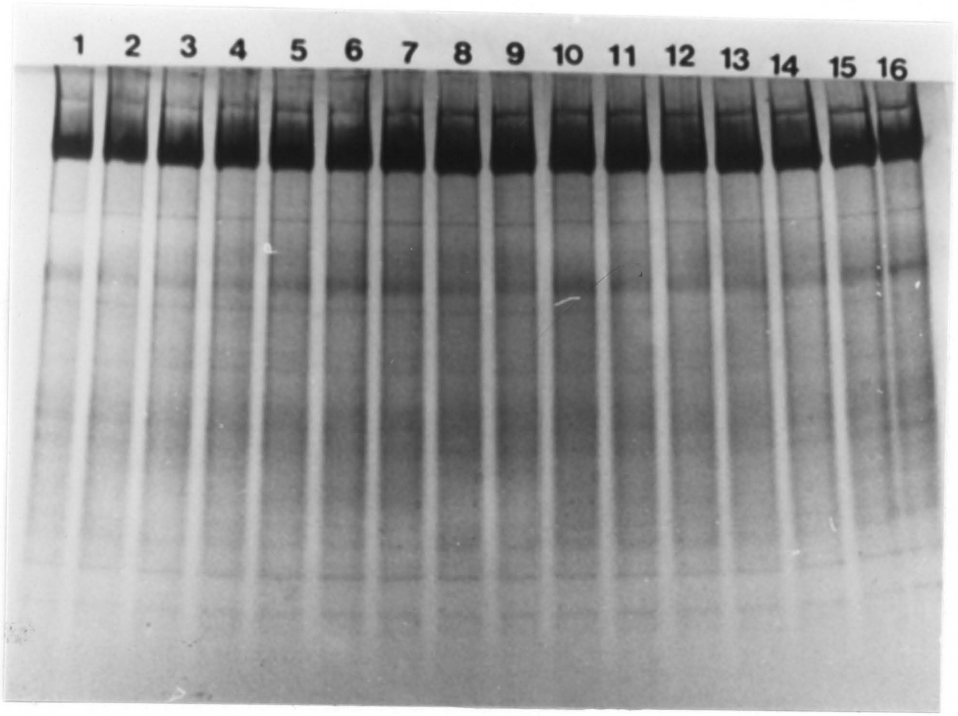


Figure 2.2. Gradient-PAGE (5 - 20 %) of leaf proteins of 16 different tomato cultivars listed in Table 2.1.

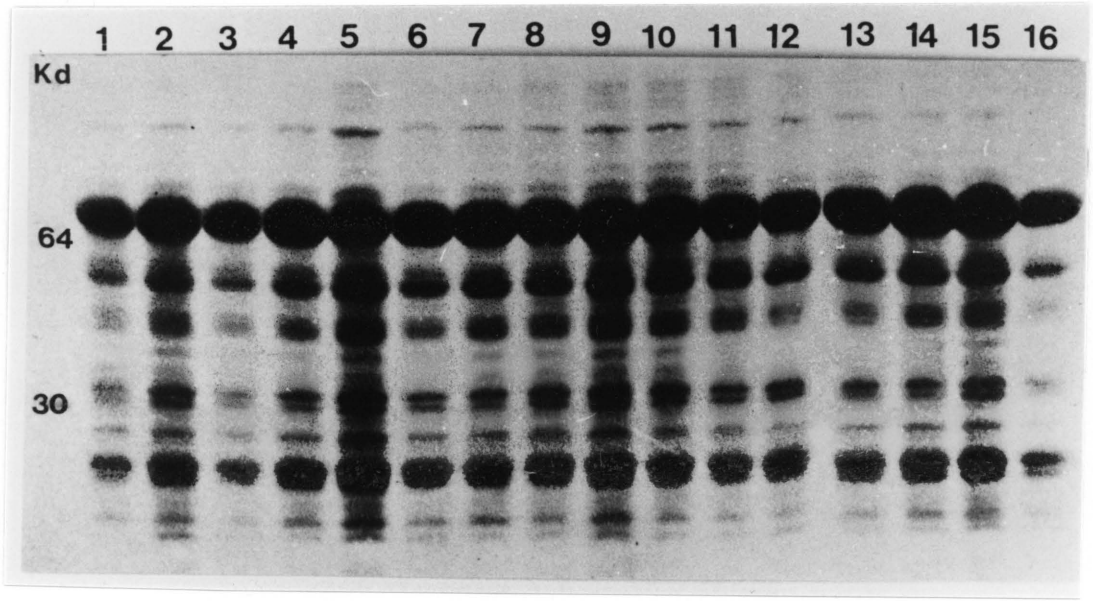


Figure 2.3. SDS-PAGE (15 %) of 16 different tomato cultivars (Table 2.1) leaf proteins.

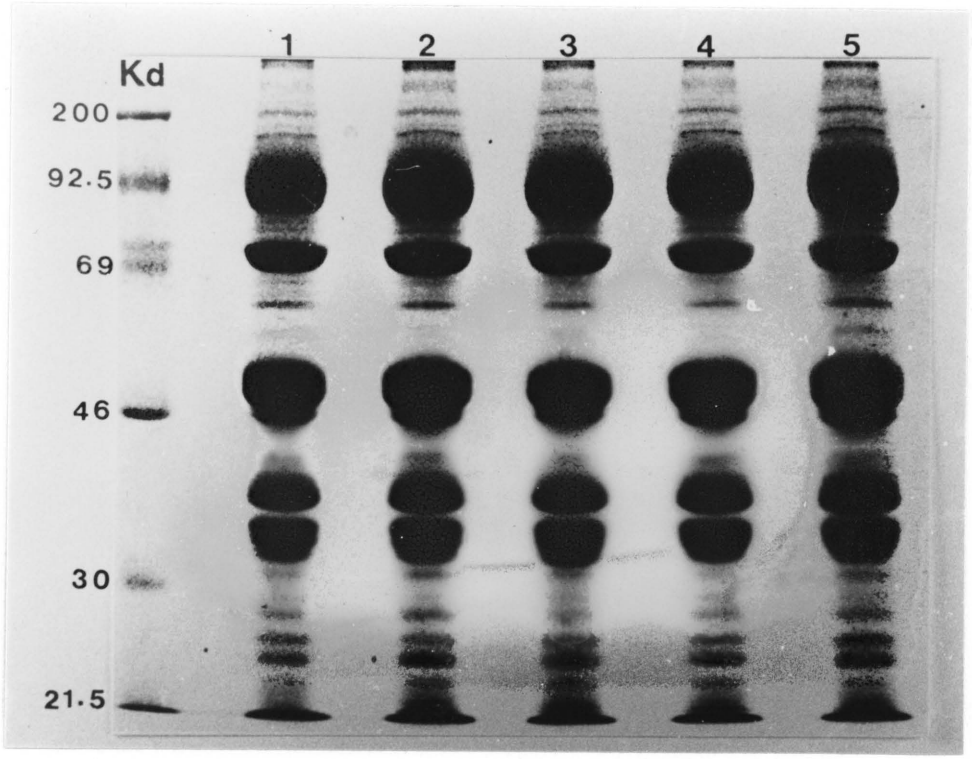


Figure 2.4. SDS-PAGE (15 %) of seed polypeptides of five tomato cultivars. Lane 1 - Rotam-4, lane 2 - Rotam-3, lane 3 - Rodade, lane 4 - Flora-Dade; lane 5 - Stevens.

Figure 2.4 shows the polypeptide profiles of tomato seeds of the first five cultivars listed in Table 2.1. The separation was performed by means of 15 % SDS-PAGE. No qualitative or quantitative differences were observed between any of the cultivars.

2.4 Discussion

The prime requirement to identify cultivars by electrophoretic techniques, is the presence of unique protein variants which are cultivar specific. In this study, the resolving power of IEF, gradient-PAGE and SDS-PAGE was compared to establish possible differences between

sixteen tomato cultivars. No apparent variation between any of these cultivars could be detected using either seed or leaf polypeptide extracts. The observed lack of diversity can be attributed to the narrow genetic base of tomato cultivars due to their self-pollinating and diploid nature, and the breeding method used to generate new cultivars (Miller and Tanksley, 1990a).

This study confirmed the work published by Sawhney *et al.,* (1985) on the occurrence of distinct protein patterns between leaves and seeds. Sawhney *et al.*, (1985) also demonstrated variation between protein patterns of floral organs of tomatoes, but no differences were observed between protein patterns within the same organ.

Native-PAGE (Stegemann, 1983) and two-dimensional gel electrophoretic methods (Padhye and Salunkhe, 1979) with silver staining (Wray *et al.*, 1981) of polyacrylamide gels can also be considered for identification of tomato cultivars, but results from this study argue against the likelihood that methods based solely on protein patterns will be able to discriminate between them.

Analysis of isozyme composition of cultivars, however, offers a refined screening method for enzyme activity rather than protein only. The electrophoretic analysis of isozymes is also uncomplicated and relatively inexpensive to perform. This topic, therefore, will be addressed in the next chapter.

CHAPTER 3

ISOZYMES AS MOLECULAR MARKERS FOR CULTIVAR IDENTIFICATION

3.1 Introduction

Isozyme analysis of tomato cultivars provides an alternative method to morphological and protein analysis for cultivar identification. Tanksley and Rick (1980) found isozyme polymorphism to be highly useful for measuring the extent of variation between different tomato species. However, isozyme variation **within** the *Lycopersicon esculentum* species was never investigated in order to determine its potential to discriminate between cultivars.

Isozymes of cultivated and wild tomatoes were mostly studied by starch gel electrophoresis (Rick and Yoder, 1988; Rick, 1983; Tanksley, *et al.*, 1982; Tanksley and Rick, 1980; Rick and Fobes, 1975). Major disadvantages of starch gel electrophoresis are the frequent lack of clarity and relative poor band resolution (Suurs *et al.*, 1989). Isozymes on starch gels are generally revealed as large blots on the gel, which may sometimes hinder unequivocal interpretation of zymograms, especially if migrational differences between isozymes are small (Oliver and Martinez-Zapater, 1985). PAGE on the contrary is transparent and yields high resolution of bands (Nielson, 1985).

Isozymes used in this study were selected for their variety in tomato cultivars. For instance, polymorphism in alcohol dehydrogenase (ADH) of tomato seeds were shown to be sufficient to analyze the purity of F1-hybrids (Van den Berg, 1991; Tanksley and Jones, 1981).

Tanksley (1979) also reported that ADH isozymes are only present in tomato seed and pollen, and probably play a role in the anaerobic respiration pathways.

Acid phosphatase (APS) isozymes displayed sufficient polymorphism to be used for screening of tomato cultivars for nematode resistance. (Ammati, 1985; Medina-Filho, 1980; Williamson and Collwell, 1991; Klein-Lankhorst, *et al.*, 1991a; Cap and Roberts, 1992). Patterson and Payne (1989) confirmed the presence of a number of APS isozymes in the roots of tomatoes.

Suurs *et al.,* (1989) reported on phosphogluco isomerase (PGI), phosphogluco mutase (PGM) and 6-phosphogluconate dehydrogenase (6-PGDH) polymorphism between *Solanum* and *Lycopersicon* species. PGM isozymes were expressed in pollen and in dry and germinating tomato seeds, while in the mature plant, PGM activity was expressed in root, stem and leaf tissue (Tanksley, 1979). Tanksley (1980) reported that PGI isozymes in dormant tomato seeds were more abundant than in imbibed seeds and that the expression of these isozymes during seed storage became constitutive after approximately 5 years. Suurs *et al.*, (1989) identified three 6-phosphogluconate dehydrogenase (6-PGDH) isozymes in tomato leaves by gradient-PAGE.

Esterase were also included in this study, since a number of different esterase isozymes were observed between male sterile and male fertile stamens of tomatoes as well as in ripe fruits (B2hadula and Sawhney, 1987).

The present study was, therefore, performed to determine if tomato isozymes (ADH, APS, PGM, PGI, EST and 6-PGDH) exhibited sufficient polymorphism to distinguish between

genetically uniform tomato cultivars. Isoelectric focusing (IEF) was used as separation method, as isozyme variants can be identified by their isoelectric points and this is likely to be more reproducible than order of mobility (Patterson and Payne, 1989). Most of these enzymes are found in a variety of plant organs, therefore leaves as well as seeds were used for isozyme analysis.

3.2 Materials and methods

Cultivars used for this study form part of the tomato breeding programme at Roodeplaat, Pretoria. The cultivars listed in Table 2.1 were used.

3.2.1 Extraction of isozymes

Collection of samples started when the first true leaves of tomato plants (16 cultivars listed in Table 2.1) reached maturity (day 0). Leaves (0.05 g) and dormant seeds (0.07 g) were crushed in liquid nitrogen with a mortar and pestle, whereafter 500 µl of the extraction buffer (Small, *et al.*, 1990) was added. The extraction buffer consisted of 0.1 M Tris-HCl pH 8.5; 0.01 M β-mercaptoethanol; 15 % (v/v) glycerol; 0.01 M sodium tetraborate; 0.002 M EDTA; 0.02 % (v/v) Triton X-100 and 5 mg/ml fatty acid-free bovine serum albumin. The crude extracts were centrifuged at 10 000 x g for 5 minutes at 4 °C and 25 µl of the supernatant was loaded per well.

3.2.2 Isoelectric focusing (IEF)

Vertical isoelectric focusing was based on the method described by Robertson *et al.* (1987) and outlined in section 2.2.3.

3.2.3 Isozyme staining

Gels were stained immediately after electrophoresis. Leaf extracts were only stained for EST due to intra-cultivar variation observed for these isozymes. Seed extracts were stained for ADH, APS, PGM, EST, PGI and 6-PGDH isozymes (Vallejos, 1983). The gels were photographed on a light box with a Polaroid direct screen instant camera (Polaroid).

The principles on which the isozyme staining are based fall into three categories (Harris and Hopkinson, 1976):

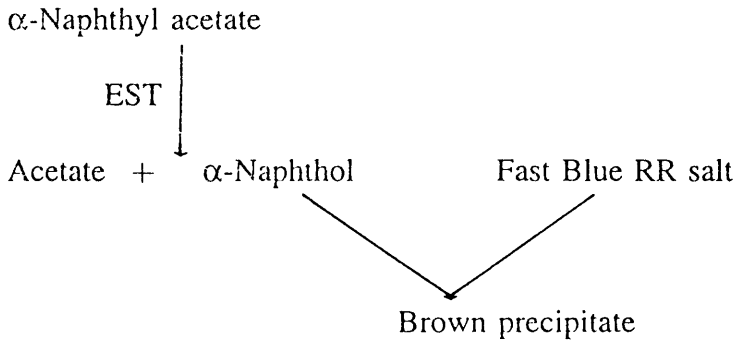
(a) The "chemical detection" staining methods, to which APS and EST isozyme staining belong. This group of staining techniques employs chemical colouring reagents (Fast Garnet GBC salt and Fast blue RR salt, respectively) to detect isozyme activity. The staining of APS and EST isozymes are "positive" staining, since the colour is produced by a reaction of the colouring agent with the product of the enzyme activity (Fig. 3.1 a).

(b) In the "electron transfer" staining method, reducing equivalents are transferred to the coenzymes NAD^+ and $NADP^+$, which in turn reduce the dyeing reagent methyl thiazolyl tetrazolium (MTT), to form an insoluble blue-purple precipitate on the gel where the isozymes

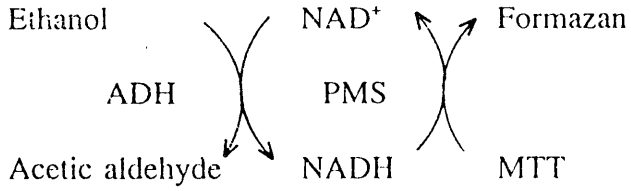
are located (Fig. 3.1 b).

(c) Isozymes of PGI and PGM are stained by the so-called "enzyme-linked" staining method. PGM and PGI staining both use glucose-6-phosphate dehydrogenase (G-6-PDH) as a linking enzyme and the specificity of each stain is detemined by the substrates used, namely fructose-6-phosphate and glucose-1-phosphate, respectively (Fig. 3.1 c). The linking enzyme changes the product of the primary enzyme reaction and together with the appropriate coenzyme ($NADP^+$), a formazan precipitate can be generated on the gel. Phenazine methosulphate (PMS) acts as a catalyst.

a.



b.



c.

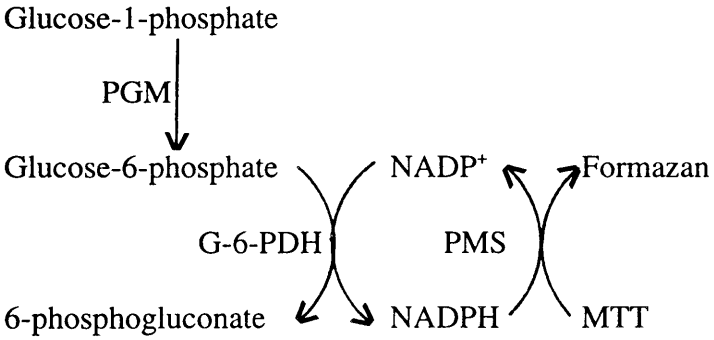


Figure 3.1 a-c. Principles of specific isozyme staining: (a) Chemical detection, (b) electron transfer (c) enzyme-linked staining methods employed for staining of eg EST, ADH and PGM isozymes, respectively.

The composition of isozyme staining reagents are summarised in Table 3.1.

Table 3.1. Composition of reagents for isozyme staining.

CHEMICAL SUBSTANCE OR BUFFER	QUANTITIES USED FOR STAINING ISOZYMES					
	ADH	**APS**	**PGM**	**EST**	**PGI**	**PGDH**
0.1 M Tris-HCl pH 7.5	100 ml		100 ml	100 ml	100 ml	100 ml
0.05 M Na-acetate pH 5.5		100 ml				
Ethanol	6 ml					
NAD^+	30 mg					
PMS[1]	4 mg		4 mg		4 mg	4 mg
MTT[2]	20 mg		20 mg		20 mg	20 mg
ß-naphthyl phosphate		30 mg				
1.0 M $MgCl_2$		1 ml	1 ml		1 ml	1 ml
Fast Garnet GBC-salt		100 mg				
Glucose-1-phosphate			150 mg			
6-PGDH			40 U		20 U	
$NADP^+$			15 mg		20 mg	15 mg
α-naphthyl acetate				50 mg		
Fast blue RR salt				100 mg		
Fructose-6-phosphate					80 mg	
6-Phosphogluconate						20 mg

[1] PMS - phenazine methosulphate
[2] MTT - methyl thiazolyl tetrazolium

3.3 Results

3.3.1 Leaf isozymes

Extracts were made immediately after collection and isozymes were separated by IEF. The same procedure was also followed three days and again six days later. Minor polymorphism were observed between leaves collected on day 0 for the 16 cultivars, except for cultivar 2 where the thick band at pI 7.5 was absent (Figure 3.2 a), Polymorphisms observed are quantitative rather than qualitative.

Figure 3.2 (b) represents EST isozyme patterns which were expressed 3 days later in the same cultivars. The thick band at pI 7.5 was either absent or the intensity was decreased. Cultivar 2, however, now showed a distinct band in this position. More polymorphisms between cultivars was detected during this period, with both qualitative and quantitative differences. However, the overall activity of esterases were decreased during this period and cultivars 3, 6, 12 and 13, hardly expressed any esterase isozymes. The only cultivars showing an increase in the number of isozyme bands, were cv's 1, 4 and 8.

Esterase isozymes expressed six days later are shown in Figure 3.2 (c). Cultivars displayed major polymorphisms during this period, except for eg. cv's 3, 5, 7, 15 and 8 and 9, respectively, which showed similar banding patterns. The band intensity and number of esterase isozymes in eg cv's 1 and 4 were decreased in comparison to the previous period, while the number and intensity of isozymes increased in eg cv 3, 6, 8, 9 and 12. Cultivars 2, 10, 11, 13 and 14 hardly show any esterase activity during this period.

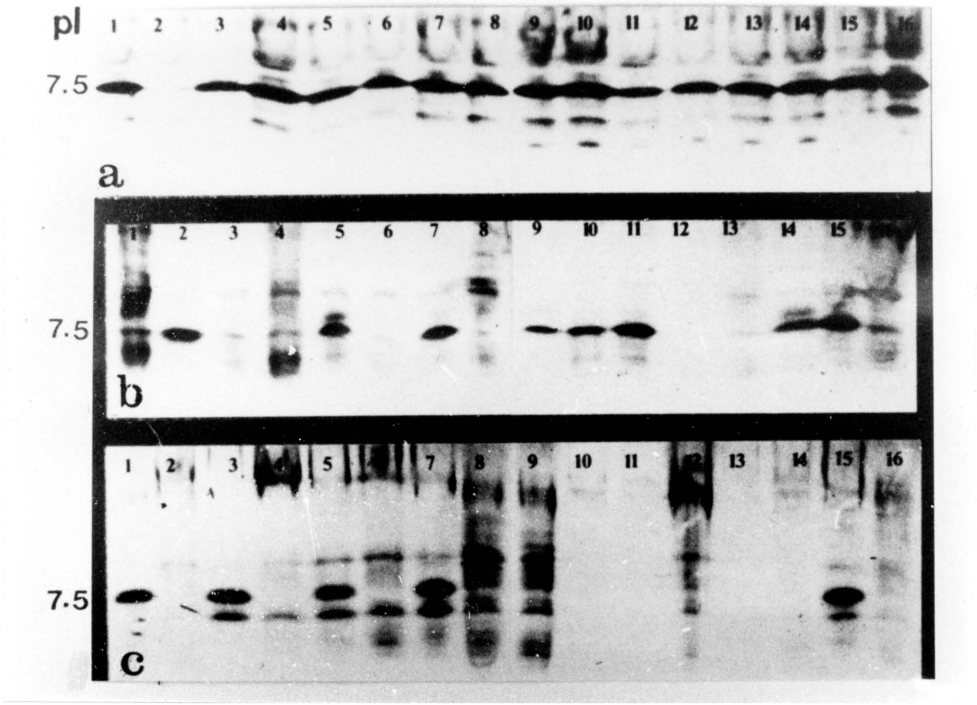


Figure 3.2 a, b and c. EST isozymes extracted from tomato leaves and separated by IEF (pH 3-10). (a) - Day 0, (b) - Day 3 and (c) - Day 6. Lanes 1 - 16 are the cultivars as indicated in Table 2.1.

3.3.2 Isozymes from seeds

Isozymes from tomato seeds were separated by vertical IEF with a narrow pH-gradient (pH 4 - 6). Cultivars were grouped into different phenotypes based upon their isozyme banding pattern. Results are described below and summarised in Table 3.1 and Figure 3.3.

Alcohol dehydrogenase (ADH). The 16 cultivars displayed nine different banding patterns, numbered A1 to A9 for ADH (Fig. 3.3). Qualitative and quantitative differences were evident.

Acid phosphatase (APS). APS isozymes could be classified into four different phenotypes, namely B1 to B4 (Fig. 3.3). Cultivars belonging to phenotype B2 (Table 3.2) were subdivided into phenotypes B2i or B2ii due to the inconsistent presence of one band at pI 5.4 (arrow; Fig. 3.3). Phenotype B1 showed an additional isozyme band (open arrow head; Fig 3.3), which distinguished it from phenotype B2. Phenotypes B3 and B4 differed qualitatively from phenotypes B1 and B2.

Phosphogluco mutase (PGM). PGM isozymes showed a maximum of four bands (Fig. 3.3), representing three different isozyme phenotypes (C1 to C3). The C2 phenotype (cv. Karino and M88/44) showed a quantitative difference, (closed arrow head; Fig. 3.3) and phenotype C3 showed a qualitative difference, (open arrow head; Fig. 3.3) characteristic of the cultivar M88/68.

Esterase (EST). Cultivars could be grouped into two EST phenotypes, distinguished from each other by a qualitative difference (open arrow head; Fig. 3.3). Phenotype D2 was characteristic of the cultivars Walter and Flora-Dade, while the remaining cultivars were represented by phenotype D1.

Phosphogluco isomerase (PGI). Two different PGI phenotypes were observed among cultivars (Fig. 3.3). The cultivars NC EBR-2 and Dawie belong to phenotype E2. Cultivars belonging to E1 had an extra isozyme band at the anodal side of the gel (arrow; Fig 3.3). Five cultivars, indicated in Table 3.2, either had phenotype E1 or E2.

6-Phosphogluconate dehydrogenase (6-PGDH). Six 6-PGDH isozymes could be separated (Fig. 3.3). Only cultivar Dawie could be distinguished from other cultivars by a qualitative difference (closed arrow head; Fig 3.3).

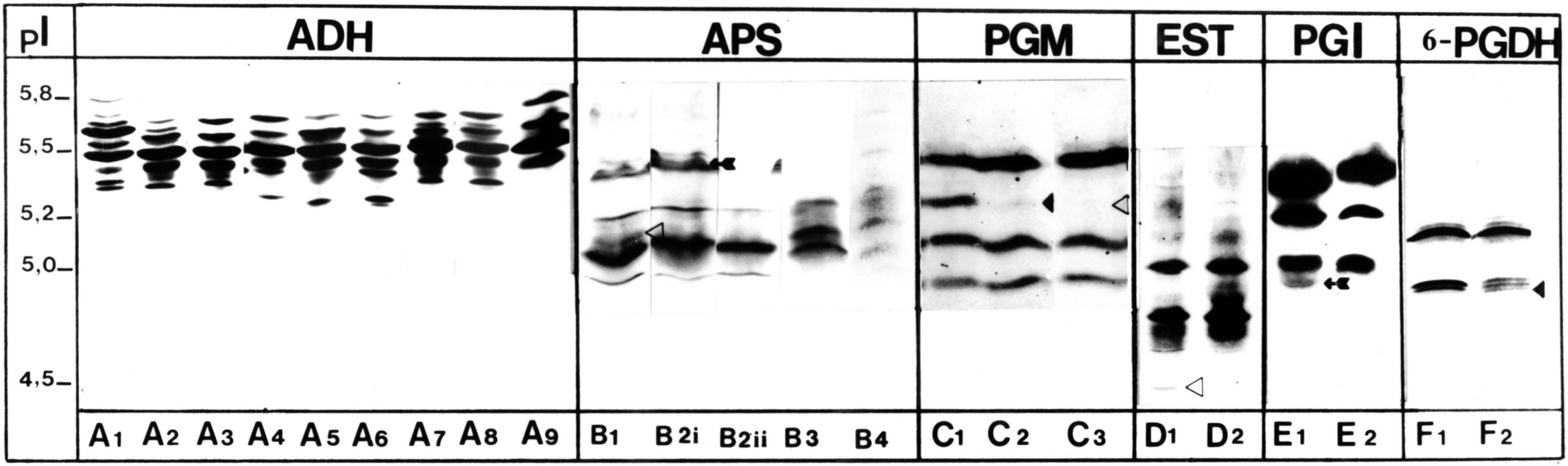


Figure. 3.3. Isozyme phenotypes separated by polyacrylamide IEF (pH4 - 6). Cultivars were grouped according to their isozyme banding patterns. Results are from quadruplicate experiments for each isozyme and cultivar. Open and closed arrowheads denote qualitative and quantitative differences, respectively; the arrows indicate inconsistent isozyme bands.

Table 3.2. Isozyme phenotypes of tomato cultivars. Each symbol describes an isozyme phenotype.

CULTIVARS	ENZYMES[1]					
	ADH	APS	PGM	EST	PGI	PGDH
Rotam-3	A1	B1	C1	D1	E1	F1
NC EBR-2	A1	B2[4]	C1	D1	E2	F1
NC EBR-1	A1	B3	C1	D1	E1	F1
Rotam-4[2]	A2	B2[4]	C1	D1	E1	F1
Rodade[2]	A2	B2[4]	C1	D1	E1	F1
Stevens[2]	A2	B2[4]	C1	D1	E1	F1
Flora-Dade	A3	B2[4]	C1	D2	E1	F1
Dawie	A4	B4	C1	D1	E2	F2
Karino	A5	B1	C2	D1	E1/2	F1
Walter	A5	B2[4]	C1	D2	E1/2	F1
L1051[3]	A6	B3	C1	D1	E1/2	F1
Ld2048[3]	A6	B3	C1	D1	E1/2	F1
Corde'oc	A6	B4	C1	D1	E1/2	F1
Irat L3	A7	B2[4]	C1	D1	E1	F1
M88/68	A8	B3	C3	D1	E1	F1
M88/44	A9	B3	C2	D1	E1	F1

[1] See Fig. 3.3 for phenotypes A to F.

[2] Cultivars with the same phenotype.

[3] Cultivars with the same phenotype, but different from 2.

[4] Phenotypes were subdivided into B2i and B2ii (Fig. 3.3) due to the inconsistency of one band.

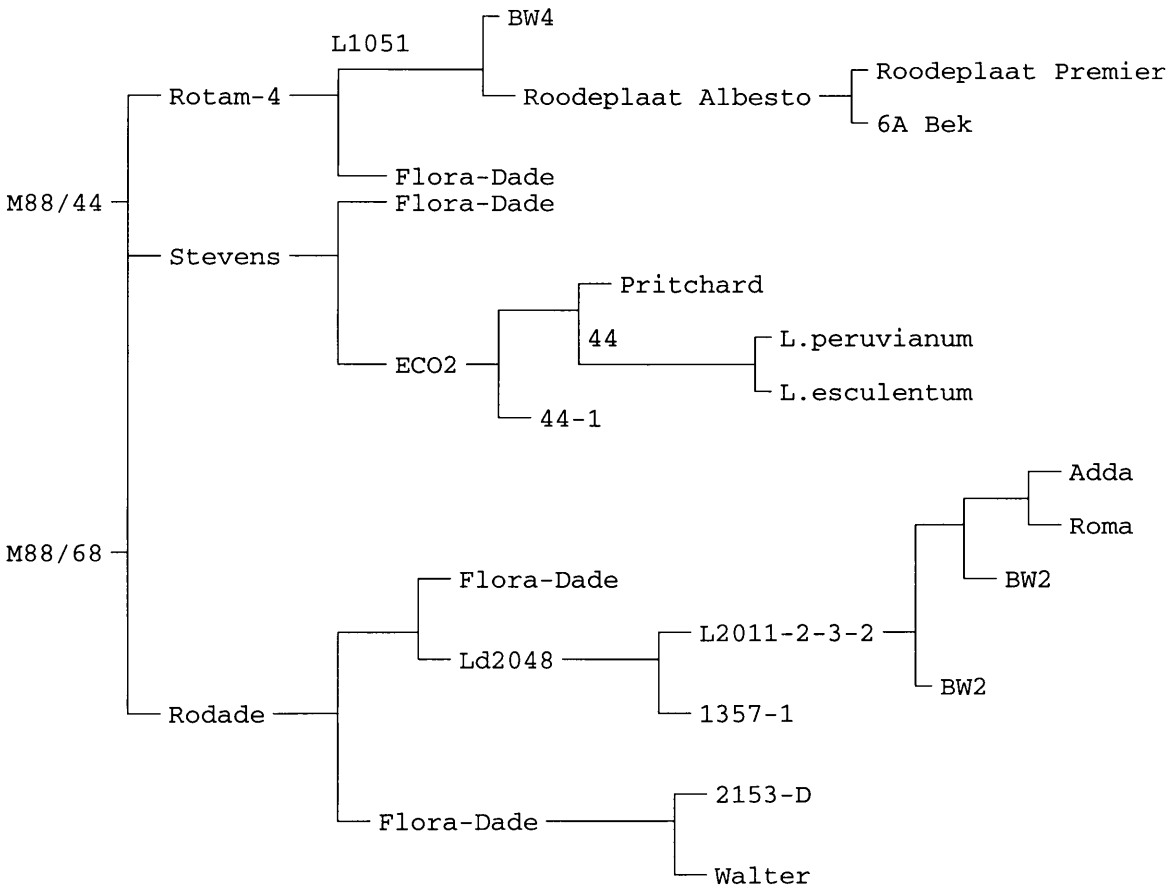


Figure. 3.4. Pedigrees of some tomato cultivars compiled from data obtained from Rotam-4 (Bosch *et al.*, 1990), Rodade (Bosch *et al.*, 1985), Flora-Dade (Volin and Bryan, 1975), Stevens, L1051, Ld2048, M88/44 and M88/68 (Unpublished data).

3.4 Discussion

Polymorphism noted in esterase isozymes of leaves could be useful to distinguish between tomato cultivars. Unfortunately, as shown in this study the expression of different esterase

isozymes varied over a period of days (Fig. 3.3) and even hours (not shown). This inconsistency of esterase isozyme expression was also noticed by Stein (1983) during fruit ripening stages of tomatoes. Esterase isozyme bands varied in number and intensity among stages, but no definite trend could be established. Bhadula and Sawhney (1987), found the same inconsistent pattern of esterase isozyme expression in a study of the ontogeny of the stamens of tomatoes. A possible explanation for this phenomenon is that the esterase isozymes have distinct functions, which are expressed according to cell demands.

More polymorphism was evident amongst seed isozymes than has been reported previously (Van den Berg, 1991; Tanksley and Jones, 1981), when isoelectric focusing with a narrow pH gradient was used (Fig. 3.3). Genetic polymorphism within two of the six enzymes were sufficient to identify the majority of cultivars based mainly on qualitative differences. Nine ADH phenotypes were distinguished and together with APS allowed the identification of 12 of the 17 cultivars (Table 3.2). The other four enzymes investigated showed less polymorphism and did not assist in the identification of additional cultivars. PGM isozymes had three different phenotypes and EST, PGI and 6-PGDH only two each (Table 3.2).

The inconsistency of the one APS band may be due either to a low concentration or instability of this isozyme. This phenomenon is also observed in some PGI phenotypes. These bands, however, were not required for cultivar identification.

Cultivars Rotam-4, Rodade and Stevens could not be distinguished from each other by any of the isozymes investigated (Table 3.2). These three cultivars have Flora-Dade, Ld2048 and L1051 as breeding parents (Fig. 3.4). It is of significance, therefore, that the latter cultivars

are distinguishable from their progeny by ADH (Flora-Dade) and ADH and APS phenotypes (Ld2048 and L1051). Cultivars Ld2048 and L1051 were indistinguishable from each other, but belong to a different phenotype than Flora-Dade (Fig. 3.3).

These results show that isoelectric focusing of tomato seed isozymes, is a promising method to discriminate between tomato cultivars which are not closely related such as L1051 and Ld 2048 (Fig. 3.4). However, to be able to distinguish between closely related cultivars such as Rotam-4, Stevens and Rodade which have Flora-Dade as a common ancestor, more enzymes need to be investigated to evaluate the potential of the method to discriminate between closely related cultivars.

Random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) are based on the screening of the whole genome of a plant in search for polymorphism. RAPD's as potential tool for discriminating between tomato cultivars is presented in the next chapter.

CHAPTER 4.

RANDOM AMPLIFIED POLYMORFIC DNA (RAPD) MARKERS FOR CULTIVAR IDENTIFICATION

4.1 Introduction

Developing highly reliable and discriminating methods for identifying cultivars has become increasingly important to plant breeders and seed companies who need sensitive tools to differentiate among cultivars for plant patent protection. Until now, tomato cultivars were identified primarily based on horticultural, morphological and physiological descriptions which may vary considerably due to environmental fluctuation and human judgement. Tomato cultivars lack distinguishing morphological characteristics due to extensive in-breeding methods (Miller and Tanksley, 1990b) and require more sensitive methods for their identification. As an addition to morphological and physiological methods, identification of some tomato cultivars based on isozyme patterns have been evaluated in chapter 3. Since isozyme polymorphism is limited to expression of structural genes, the number of loci that can be resolved is limited and all the genetic differences among cultivars may not be visualized. These restrictions may be overcome by the use of DNA based methods, which are independent of environmental conditions, plant tissue or developmental stages or number of loci detected.

The most widely used DNA based technique for cultivar identification of different crops is RFLP's. Broun *et al.* (1992) used a telomeric probe (terminal 7-base-pair tandem repeat area

of approximately 20 -50 kb) and a TGRI probe (closely linked 162-bp subterminal tandem repeat of 100 - 1000 kb) to distinguish between tomato cultivars, but a mutation rate of 2 % per generation in these parts of the chromosome lessens the usefulness of these RFLP probes. More recently, the random amplified polymorphic DNA (RAPD) technique (Williams, *et al.*, 1990) based on the polymerase chain reaction (PCR), has been developed to detect polymorphism in some species (Welsh *et al.*, 1991; Welsh and McClelland, 1990). The technique employs a single oligonucleotide primer of ± 10 bases long to generate DNA fingerprints. This technique is highly sensitive with regard to nucleotide differences between primer and the template (Williams *et al.*, 1991). Klein-Lankhorst *et al.* (1991b) reported that a change of one nucleotide in a primer resulted in amplifying a unique part of the genome. This supports the conclusion drawn by Williams *et al.* (1990) that the RAPD assay is capable of detecting single base pair changes in genomic DNA. These characteristics make RAPD's a very effective method to discriminate between species which lack polymorphism within cultivars, such as tomatoes (Williams *et al.*, 1991). RAPD markers are inherited in a Mendelian manner and are dominant. This means that only one allele at a locus is amplified and detected at a time. Therefore, the absence of a RAPD marker represents all other alleles at that locus that did not anneal with the primer. RAPD markers, therefore, cannot distinguish between heterozygotic loci (Rafalski *et al.*, 1991). The RAPD technique has similar uses as RFLP analysis, namely the construction of linkage maps, identification of cultivars and parentage determination. Advantages of RAPD's over RFLP's for cultivar identification include the following: The equipment and supplies necessary are inexpensive relative to those needed for RFLP's. The RAPD procedure can be automated, the analysis is quick, Southern blotting and labelled probes are not used and minimal quantities of DNA are needed.

Williams *et al.* (1993) indicated a number of reaction conditions which should be taken into consideration when using the RAPD procedure. An important variable is the concentration of the genomic DNA used as template. Different DNA extraction procedures produce DNA of different purity and it may be necessary to optimize the amount of DNA used in the RAPD assay to achieve reproducibility and a strong signal. Too much genomic DNA may result in smears or a lack of clearly defined bands on the gel. Too little DNA yields patterns which are not reproducible. It is also important to note that both the magnesium ion concentration and the annealing temperature affect the relative intensity of amplified bands.

Primer concentration should also be optimised, since low concentrations produce amplified bands with low detectability, while at high concentrations smearing of the bands is evident. Williams *et al.* (1993) also indicated that changes in the nucleotide sequence at the 3'-position of a primer resulted in complete changes in the banding pattern, while changes at the 5'-end had a smaller effect. They postulated that nucleotide changes in the template site will have the same effect on specificity as those observed for changes in the primer, and that the RAPD assay may therefore be used to detect single base changes in the template.

A low deoxyribonucleotide triphosphate (dNTP) concentration results in weak band intensity, but no effect on band intensity has been noted at high concentrations of dNTP's (Williams *et al.*, 1993).

The objective of this study was firstly to establish optimum conditions for RAPD analysis of tomatoes and secondly to test the discriminating power of RAPD markers to distinguish between advanced breeding lines (F10), derived from the same breeding parents. The

ancestors of these breeding parents (cultivars) were also analysed. RAPD conditions should be optimized, since the random binding of primer to template may lead to results which are not reproducible. Conditions tested for optimum RAPD results were template DNA concentration, $MgCl_2$ concentration, the effect of block position and different primers. F1 and F2 hybrids were also included in the investigation in an attempt to trace RAPD markers in the progeny. Genomic DNA (template DNA) was extracted from tomato seeds instead of leaves to prevent chlorophyll contamination as it is a chelating agent which may scavenge Mg^{++} which is needed to stabilize primer/template hybrids.

4.2 Materials and methods

4.2.1 Plant material

Advanced breeding lines, their breeding parents (cultivars) and their F1 and F2 progeny used for RAPD analysis are listed in Table 4.1. The use of F1 and F2 progeny was an attempt to track RAPD markers in the off-spring.

4.2.2 DNA extraction

Genomic DNA of plants listed in Table 4.1 was extracted by the following: Tomato seeds (0.1 g) were crushed in liquid nitrogen with a mortar and pestle. Two millilitres of the proteinase-K extraction buffer (50 mM Tris-HCl pH 8.0; 100 mM NaCl; 10 mM EDTA; 1 % SDS; 10 mg/ml proteinase-K) was added and the solution was incubated at 65 °C for 45 minutes. The extracts were centrifuged for 10 minutes at 10 000 x g to remove cellular debris. A 5 M NaCl

solution (100 µl) was added, followed by an equal volume of chloroform:isoamyl alcohol (24:1) and centrifugation for 30 seconds at 10 000 x g. The aqueous phase was collected and 1 volume of Tris-saturated phenol (Sambrook *et al.* 1989) was added, followed by a centrifugation step (30 sec at 10 000 x g). Chloroform:isoamyl alcohol was again added to the aqueous phase and the extraction step was repeated. The organic phase was discarded and an equal volume of 96 % ethanol was added and left overnight at -20 °C to precipitate the DNA. The DNA was collected by centrifugation for 30 seconds at 4 °C. The pellet was washed twice with 70 % ethanol, dried and resuspended in 100 µl sterile water.

Contaminating RNA was removed by addition of 5 µl of a 500 µg/ml stock solution of RNase and left overnight at room temperature (Deragon and Landry, 1992).

Table 4.1. Advanced breeding lines, cultivars and F1 and F2 hybrids used for RAPD analysis.

DESCRIPTION	NAME	CROSSING
adv. breeding line[1]	M88/68	Stevens x Rodade
adv. breeding line	M88/75	Stevens x Rodade
adv. breeding line	M88/87	Stevens x Rodade
adv. breeding line	M88/54	Stevens x Rotam-4
adv. breeding line	M88/37	Stevens x Rotam-4
cultivar	Rotam-4	parent: adv. breeding lines
cultivar	Rodade	parent: adv. breeding lines
cultivar	Stevens	parent: adv. breeding line and F1, F2
cultivar	Flora-Dade	parent: F1 and F2 progeny
F1 progeny		Flora-Dade x Stevens
F2 progeny		Flora-Dade x Stevens

[1] advanced breeding line (F10)

DNA quality was assessed by agarose gel electrophoresis and ethidium bromide staining (Sambrook *et al.* 1989). A 0.75 % agarose gel in TAE-buffer (0.04 M Tris-acetate; 0.001 M EDTA, pH 8.0) was used.

DNA concentrations were fluorometrically determined with a TKO100 DNA fluorometer (Hoefer). Two microliters of the DNA sample was added to 2 ml working solution (1 x TNE solution (10 mM Tris-HCl; 0.2 M NaCl; 1 mM EDTA); 1 mg/ml Hoechst 33258). The concentration was determined using a 1 µg/ml calf thymus DNA standard (Operating Instructions TKO 100 Mini-Fluorometer, Hoefer Scientific Instruments).

DNA concentrations were also measured with a spectrophotometer at 260 nm and the purity (protein contamination) of the samples were assessed from the 260 nm:280 nm ratio (Sambrook *et al.*, 1989).

4.2.3 Polymerase chain reaction (PCR)

All PCR's were performed with a Hybaid dry thermal cycler (SA Scientific, SA). The RAPD method was optimized with respect to:

(a) DNA concentration

The DNA concentration for tomato RAPD's was optimized by using six different concentrations (25 ng, 50 ng, 100 ng, 200 ng, 300 ng and 400 ng), while other conditions were kept constant (section 4.3.3 (d), primer OPA-01).

(b) $MgCl_2$ concentration

Optimum $MgCl_2$ concentrations were determined by keeping the PCR conditions as described in paragraph 4.3.3 (d) (primer OPA-19) constant, but varying the $MgCl_2$ content from 0.5 mM to 4.0 mM with intervals of 0.5 mM.

(c) Position of test tubes

The position of test tubes on the PCR heating block was tested for the effect on reproducibility of results. The same reaction conditions (paragraph 4.3.3 (d), primer OPA-19) were used for five tubes placed in different positions on the heating block, namely top left, top right, bottom left, bottom right and middle.

(d) Primers

Primers used for RAPD's were obtained from Operon (SA Scientific Products). Their sequences and percentage guanine/cytosine content are shown in Table 4.2.

Table 4.2. Sequences and guanine/cytosine content of primers used for RAPD analysis.

PRIMER	SEQUENCE	PERCENTAGE G/C
OPA-1	CAGGCCCTTC	70
OPA-2	TGCCGAGCTG	70
OPA-3	AGTCAGCCAC	60
OPA-4	AATCGGGCTG	60
OPA-5	AGGGGTCTTG	60
OPA-19	CAAACGTCGG	60

4.3 Results

4.3.1 DNA extraction

Figure 4.1 shows an agarose gel of DNA extracted with the Proteinase-K extraction method before RNase treatment. The open and closed arrow head indicate genomic DNA and total RNA, respectively. No degraded DNA was noticed on the gel.

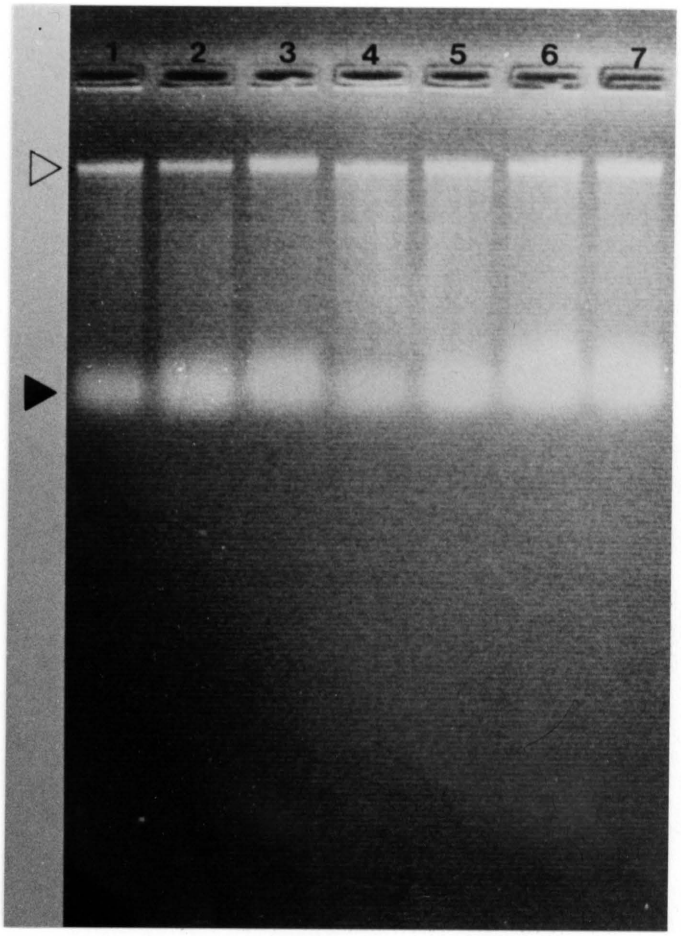


Figure 4.1. Genomic DNA extracted from seeds. The open arrow head indicates the DNA and the closed arrow head the RNA. Lanes 1 - 7 are DNA extracted from the first 7 plants in Table 4.1.

4.3.2 Determination of DNA concentrations

The DNA concentrations determined fluorometrically are shown in Table 4.3. The fluorochrome (Hoechst) used for the determinations binds preferentially to the A/T (Adenine-Thymine) rich regions of the DNA (Operating Instructions TKO 100 Mini-Fluorometer, Hoefer). No RNA or protein contamination in a DNA sample is therefore detected by this method, in contrast to spectrophotometrical determinations where DNA, RNA and protein absorb at 260 nm.

In a DNA sample with little or no protein contamination, the ratio of the absorbances taken at 260 and 280 nm should be 1.8 (Sambrook *et al.*, 1989). Moderate contamination was detected in these samples (Table 4.3). The fluorometric method was used to calculate DNA concentrations for RAPD analysis.

Table 4.3. DNA concentrations and protein contamination (A_{260}:A_{280} ratio) of DNA samples.

CULTIVARS/BREEDING LINES/HYBRIDS	µg DNA[1] / ml extraction buffer	A_{260}:A_{280}[2]
M88/68	108	1.4
M88/75	118	1.3
M88/87	128	1.4
M88/54	143	1.4
M88/37	116	1.4
Rotam-4	69	1.3
Rodade	100	1.3
Stevens	93	1.3
Flora-Dade	80	1.4
F1 hybrid	105	1.3
F2 hybrid	56	1.3

[1] DNA concentrations from 0.1 g seeds as fluorometrically determined.

[2] Indication of the purity of DNA samples.

4.3.3 PCR conditions

(a) Effect of DNA concentrations

Figure 4.2 shows the effect of different DNA concentrations (25, 50, 100, 200, 300 and 400 ng) on RAPD results using primer OPA-1 (50 ng) and cultivar Rodade. PCR conditions described in paragraph 4.3.3 (d) were used. No difference in the number of RAPD bands was noted under any of the reaction conditions. The fine RAPD band marked with an arrow in lane 1 (Fig. 4.2) was not reproducible in a repetition of the experiment. RAPD band intensities at DNA concentrations of 25, 50 and 100 ng (lanes 1 - 3, respectively) were similar but decreased slightly at higher DNA concentrations (200, 300 and 400 ng).

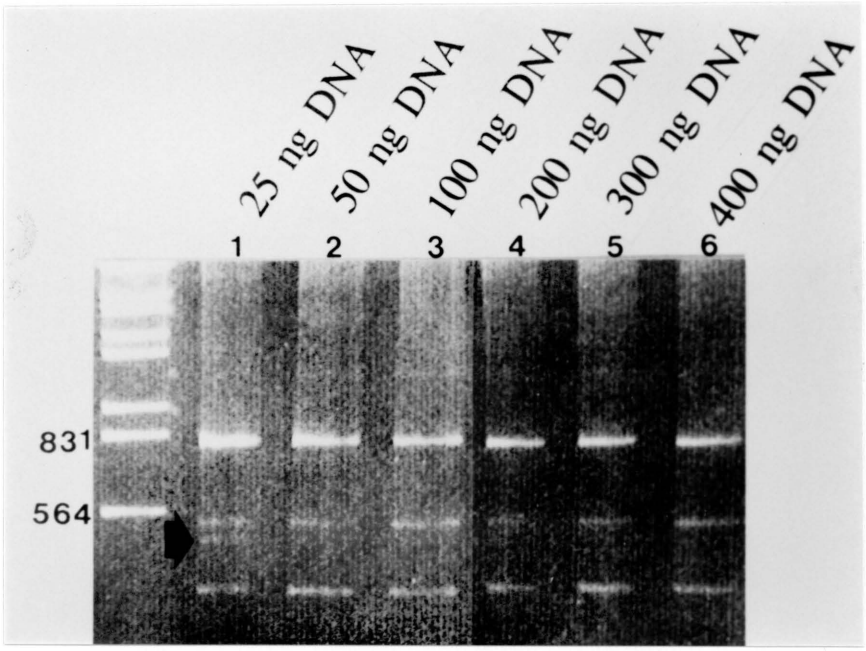


Figure 4.2. The effect of different DNA template concentrations on RAPD results separated by 10 % PAGE. Primer OPA-1, DNA from Rodade and reaction conditions described in paragraph 4.3.3 (d) were used. Lane 1 - 25 ng, lane 2 - 50 ng, lane 3 - 100 ng, lane 4 - 200 ng, lane 5 - 300 ng, lane 6 - 400 ng.

(b) Effect of $MgCl_2$

The effect of $MgCl_2$ content on the generation of RAPD bands was tested with the reaction conditions described in paragraph 4.3.3 (d) (50 ng primer OPA-19 and 100 ng DNA) and $MgCl_2$ concentrations varying from 0.5 mM to 4.0 mM (Figure 4.3). No significant changes in the RAPD patterns were detected, except at the lowest $MgCl_2$ concentration (0.5 mM) where fewer clear bands were apparent. Intensities of the clear bands (closed arrows) in lanes 2 - 8, were the highest at $MgCl_2$ concentrations of 1.5 mM and 2.0 mM (lanes 3 and 4). The intensity of the clear band (closed arrow head) in lane 1 decreased with higher $MgCl_2$ concentrations. Fine RAPD bands have the tendency to be non-reproducible (Deragon and Landry, 1992) and, therefore the fine bands generated under these conditions were not taken into consideration to establish optimum $MgCl_2$ concentrations. Since 1.5 mM $MgCl_2$ showed the highest band intensities, this concentration was used for subsequent investigations.

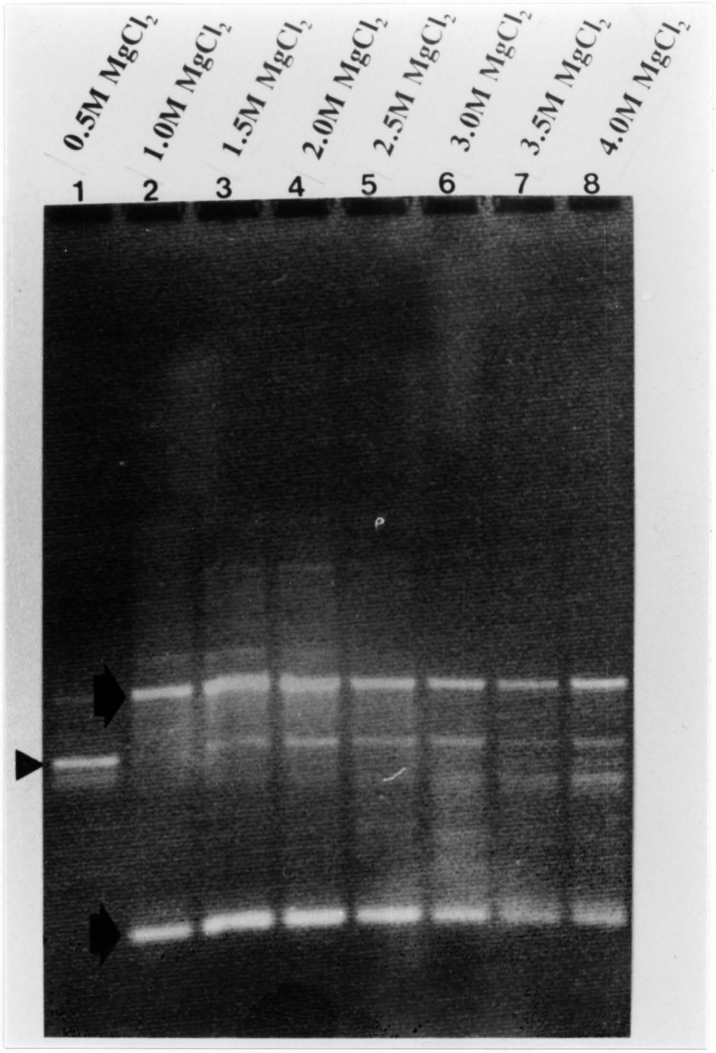


Figure 4.3. Lanes 1 - 8 shows the influence of different $MgCl_2$ concentrations on RAPD results, separated by 2 % agarose gel electrophoresis. The PCR conditions in section 4.3.3 (d), primer OPA-19 (50 ng) and DNA from Rodade (100 ng) were used. Lane 1 - 0.5 mM $MgCl_2$, lane 2 - 1.0 mM, lane 3 - 1.5 mM, lane 4 - 2.0 mM, lane 5 - 2.5 mM, lane 6 - 3.0 mM, lane 7 - 3.5 mM and lane 8 - 4.0 mM $MgCl_2$.

(c) Effect of test tube position on PCR block

An experiment was conducted to verify the uniformity of the heating block as measured by the reproducibility of RAPD results (Figure 4.4). The tubes were arranged in such a way that

tube 1 was positioned at the top left, tube 2 at the top right, tube 3 in the middle, tube 4 at the bottom left and tube 5 at the bottom right, of the PCR block. PCR conditions described in paragraph 4.3.3 (d) were used. No difference in RAPD banding patterns between any of the block positions were found (Figure 4.4). The two distinct bands (arrow heads) were reproducible, whereas the faint bands, visible in lanes 3 and 5, were not reproducible. This confirmed the hypothesis that RAPD results should be based on the distinct bands. Therefore, the position of the test tubes on the block did not influence the reproducibility of results, either because the reaction is not sensitive for slight temperature changes, or the heating of the block was uniform.

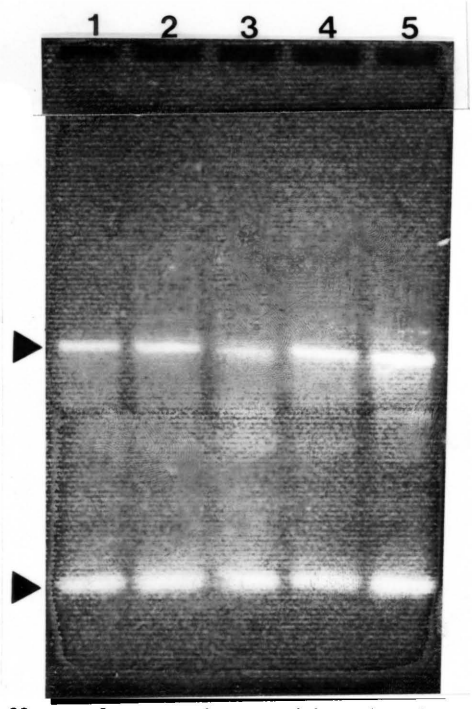


Figure 4.4. The effect of test tube position in the PCR block on RAPD results. PCR conditions in paragraph 4.3.3 (d), Rodade (100 ng) and primer OPA-19 (50 ng) were used and products were separated on a 2 % agarose gel. Lane 1 - tube 1 on the top left, lane 2 - tube 2 on the top right, lane 3 - tube 3 in the middle of the block, lane 4 - tube 4 bottom left and lane 5 - tube 5 on the bottom right of the PCR block.

(d) Optimized RAPD conditions

The optimized RAPD conditions were as follows: Samples were prepared in 20 µl, using reaction buffer (10 mM Tris-HCl pH 8.2; 50 mM KCl; 0.001 % gelatine) and 1.5 mM $MgCl_2$ (Promega), 0.1 mM deoxynucleotide triphosphate (dNTP's)(Promega), 1.0 U *Taq* DNA polymerase (Promega; one unit is defined as the amount of enzyme required to catalize the incorporation of 10 nmol of dNTP into an acid-insoluble form in 30 minutes at 74 °C), 50 ng primer and 100 ng template DNA.

One cycle at 94 °C for five minutes was followed by fifty five cycles of 1 min at 94 °C, 2 min at 35 °C; 2 min at 72 °C with an applied temperature ramp of 1 °C/3s for the 35 °C - 72 °C transition. An extra 0.5 U *Taq* polimerase was added half way through the reaction (Klein-Lankhorst *et al.*, 1991b). Genomic DNA was omitted from control reactions for each primer.

4.3.4 RAPD profiles

Thermal cycling conditions, optimized in the previous experiments, were used for the comparison of RAPD profiles of tomato cultivars and breeding lines. The conditions, as detailed in paragraph 4.3.3, are 100 ng of the template DNA, 50 ng primer, 1.5 mM $MgCl_2$ and reaction buffer. Plants and primers (OPA-1 - OPA-5) used for this study are listed in Tables 4.1 and 4.2, respectively. The first two primers had a G/C content of 70 % and the last three 60 %. The results are depicted in Figure 4.5 a - e after separation of RAPD bands on 10 % PAGE, except for Figure 4.5 (b), where a 2 % agarose gel was used.

Figure 4.5 (a) shows three distinct bands (arrows) that were reproducible in quadruplicate experiments, with primer OPA-1. In lane 7 (Rodade) one of the distinct bands was fainter than the others (arrow head), but was not be considered as a polymorphism, since this band was present in the rest of the quadruplicates. No differences in banding patterns were apparent between breeding lines, cultivars, F1 and F2 hybrids.

RAPD bands created with primer OPA-2 are shown in Figure 4.5 (b). Only one distinct band of 564 base pairs was obtained. Although this band was absent for Flora-Dade and F1 hybrid in lanes 9 and 10 it was present in the other repetitions. Thus, no differences were found between the plants listed in Table 4.1.

No clear, reproducible bands was obtained with primer OPA-3 (Figure 4.5 c).

One clear and reproducible RAPD band per plant was obtained with primer OPA-4 (Fig. 4.5 d). This band, consisting of less than 564 bp, is distinct from the single band formed by primer OPA-2 (564 bp)(Figure 4.5 b), and can be assumed to represent another part of the genome. A number of faint bands is noticed in most of the lanes, but were not considered in the interpretation of the results.

Two distinct RAPD bands (arrows) were obtained with primer OPA-5. M88/68, M88/54 and the F1 hybrid do not show any RAPD bands, but bands were evident in other repetitions. The RAPD band with the highest mobility which is absent for M88/87 and Stevens was also present in other repetitions.

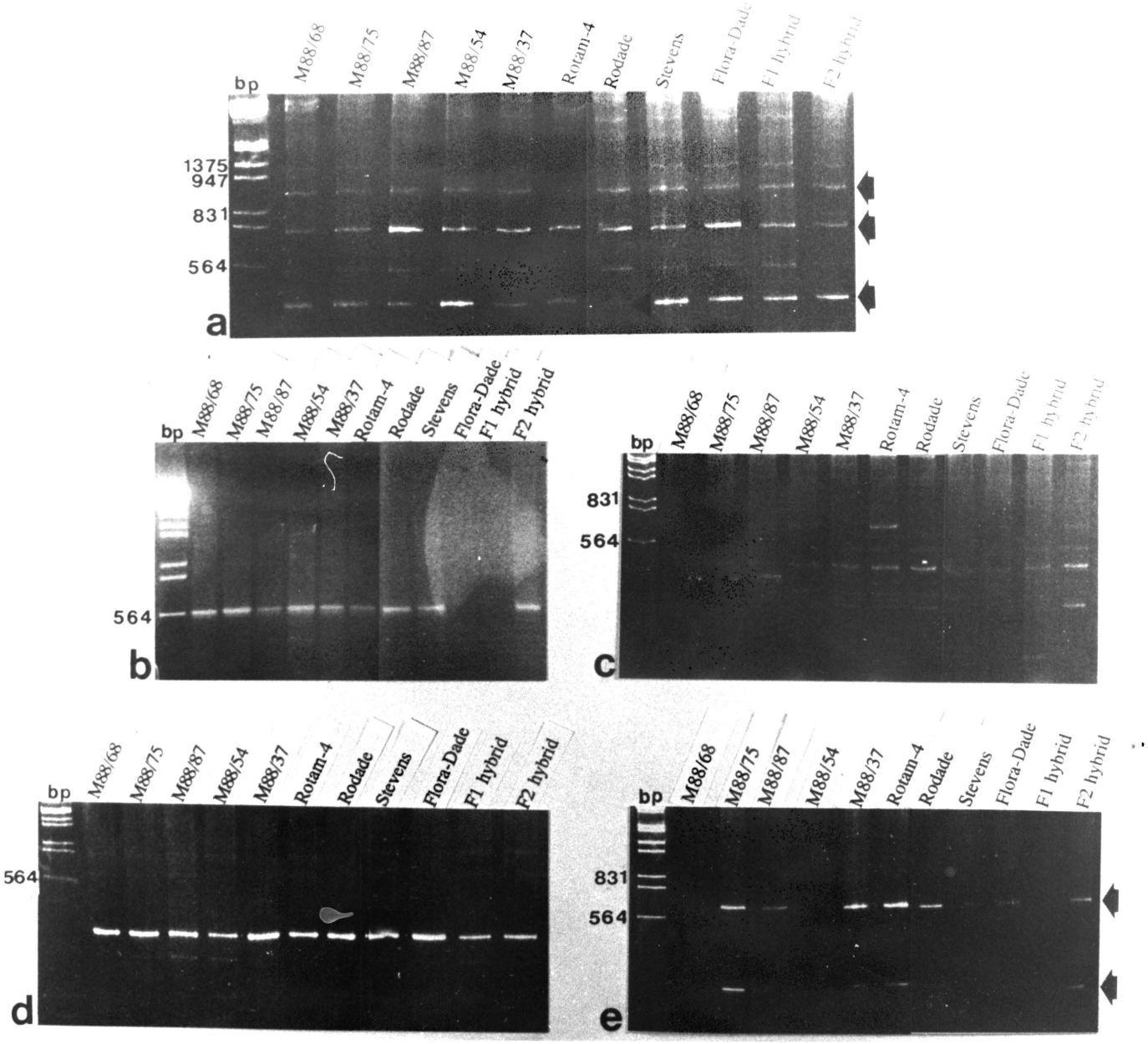


Figure. 4.5 a - e. RAPD profiles established with primers OPA-1 - OPA-5 (Figures a - e, respectively). 50 ng primer, 100 ng template DNA, 1.5 mM $MgCl_2$ and 1 x reaction buffer were used. Separation of the bands were performed by 10 % PAGE, except for Figure 4.5 (b) where RAPD bands were separated by 2 % agarose gel electrophoresis. Lane 1 - advanced breeding line M88/68, lane 2 - advanced breeding line M88/75, lane 3 - advanced breeding line M88/87, lane 4 - advanced breeding line M88/54, lane 5 - advanced breeding line M88/37, lane 6 - Rotam-4, lane 7 - Rodade, lane 8 - Stevens, lane 9 - Flora-Dade, lane 10 - F1 hybrids and lane 11 - F2 hybrids. DNA molecular markers (pGem-3, Promega) were as indicated.

4.4 Discussion

The RAPD method was independently developed during the early 90's by Williams *et al.* (1990) and Welsh and McClelland (1990). Conditions need to be optimized for the specific species under investigation and the method of Klein-Lankhorst *et al.* (1991b), was followed as a general guideline for RAPD's of tomatoes. Parameters that were investigated in this study were the effects of DNA template concentration, different primers, $MgCl_2$ concentration and position of test tubes in the PCR heating block.

DNA template concentrations were varied from 25 - 400 ng in this study and although there was slight differences in band intensities between the lower (25, 50 and 100 ng) and the higher concentrations (200, 300 and 400 ng) of DNA, the number of bands did not change (Fig. 4.2). Whereas 100 ng was chosen for further study, Welsh and McClelland (1990) and Williams *et al.* (1990) established DNA template concentrations of up to 15 ng as optimal for RAPD's of bacterial and soybean DNA. Klein-Lankhorst *et al.* (1991b) used DNA concentrations of 200 ng for their studies on tomatoes. However, it is unknown if a spectrophotometric method was used by Klein-Lankhorst *et al.* (1991b) to determine their DNA concentrations. The fluorometric method, employed in this study, detects only DNA compared to the spectrophotometric method which measure DNA, RNA, as well as proteins in a sample.

The optimization of Mg^{++} concentrations in the RAPD reaction is crucial as it enhances the stability of primer/template interactions and hence the reproducibility of bands (Williams, *et al.*, 1991b). At lower concentrations of $MgCl_2$ (1.0 - 2.0 mM) the intensity of clear RAPD

bands was higher than that at higher concentrations (2.5 - 4.0 mM)(Fig. 4.3, lanes 2 - 8). Williams, *et al.* (1993) reported that increased $MgCl_2$ concentrations resulted in more efficient amplification of some DNA segments, while others were amplified less efficiently. In this study the intensities of RAPD bands declined with an increase in $MgCl_2$ concentration. Williams, *et al.* (1993) also did not find any variation in the number of RAPD bands with varying concentrations of $MgCl_2$, whereas in this investigation the lowest $MgCl_2$ concentration (0.5 mM) resulted in the priming of different bands (Fig. 4.3). A $MgCl_2$ concentration of 1.5 mM resulted in the highest band intensity and was thus used for experiments to compare RAPD bands between cultivars. Williams, *et al.*, (1993) reported that deoxynucleotide triphosphate (dNTP's) concentrations, within a reasonable range, has no effect on relative band intensity and optimum concentrations were therefore not established in this study.

Five distinct profiles of tomato cultivars were obtained with five different random decamer primers. The substitution of one or more nucleotides in a primer resulted in the amplification of unique banding patterns, probably due to the amplification of different parts of the genome. Primers varied in their ability to amplify RAPD bands. Primers OPA-2 and OPA-4 primed only one band each, primers OPA-1 and OPA-5 amplified three and two bands, respectively, while primer OPA-3 failed to produce any clear reproducible band (Fig. 4.5). These results show that not every random decamer primer has an equal ability to prime genomic DNA and a large number needed to be employed to select suitable primers. This conclusion is supported by Martin *et al.* (1991) who neededd 144 primers to identify the *Pto* gene (resistance to *Pseudomonas syringae*). The G/C content of a primer plays a significant role in producing RAPD bands. At least four guanine or cytosine nucleotides in a 10-mer primer compared to five G's and C's in a 9-mer primer are required to create RAPD bands (Williams *et al.* 1990).

The number of RAPD bands obtained are furthermore directly proportional to the G/C content of a primer (Williams *et al.*, 1993), but no differences in number of RAPD bands were observed between primers containing 60 or 70 % G's and C's. In this study the number of RAPD bands also cannot be correlated with the G/C content between the 60 or 70 % primers.

Optimum conditions resulted in the generation of reproducible and clear RAPD bands. However, frequently no bands were amplified under the same conditions (Fig. 4.5 b, lanes 9 and 10; Fig. 4.5 e, lanes 1, 4 and 10). The temperature across the thermocycler block was apparently uniform and can be excluded as a possible cause. It is possible that 2other factors interfered with the annealing of the primer to the template, and thus the extent of template amplification. The complexity of the amplification procedure, poor discrimination by a primer between alternative priming sites of slightly different nucleotide sequences or the use of less purified DNA may explain these inconsistencies. Feasible solutions for this problem could be to use primers with higher G/C contents (higher than 70 %) or/and to purify the template DNA by including a cesium chloride purification step as suggested by Deragon and Landry, (1992). The non-reproducibility of faint RAPD bands, do not effect the usefulness of the procedure as they were normally ignored when results were interpreted. Faint bands also do not segregate in a Mendelian manner and therefore, cannot be used in genetic mapping (Deragon and Landry, 1992).

Welsh and McCleland (1991) stated that a difference of one nucleotide on the template of two individuals resulted in different RAPD patterns. As no differences between cultivars and advanced breeding lines (Table 4.1) were detected by one of these primers one can assume that the parts on the genome primed by these primers were similar. Cultivars and advanced

breeding lines used in this study are genetically closely related (Table 4.1). The advanced breeding lines were bred from the same parents, namely Stevens, the female parent, and Rodade and Rotam-4 the male parents (Table 4.1). Stevens, Rodade and Rotam-4 are also closely related and could not be distinguished by means of isozyme analysis (Chapter 3, Fig. 3.3). No differences between F1 hybrids, F2 hybrids or their parents (Flora-Dade and Stevens) could be established, therefore, it was impossible to verify if RAPD bands from the parents were inherited by the off-spring.

The possibility to discover polymorphism between genetically related individuals may improve if the number of random primers is increased. Since the annealing of primers to template is random, there is no control to which part of the genome it will bind. Silver staining is another way of visualising more RAPD bands, but it will probably be the faint bands that become more clear and one can predict that these bands will be non-reproducible. RAPD bands, employed as RFLP probes, Southern blot analysis of RAPD bands and restriction enzyme digestion of DNA before RAPD analysis can also be implemented to improve the possibility to detect polymorphism.

RAPD has recently been applied as molecular markers linked to economical important genes, such as nematode resistance in tomatoes (Klein-Lankhorst *et al.* 1991a). However, since a linkage between nematode resistance and the Aps-1^1 isozyme were reported for tomatoes, it was deemed necessary to first confirm this linkage for local cultivars before proceeding in the development of RAPD's as molecular markers. This topic is discussed in the next chapter.

CHAPTER 5.

INVESTIGATION OF THE LINKAGE BETWEEN THE ACID PHOSPHATASE ISOZYME (Aps-1^1) AND THE NEMATODE RESISTANT GENE (*Mi*).

5.1 Introduction

Infection with root knot nematodes (*Meloidogyne* spp.) is an important cause of tomato crop losses in South Africa. Infested plants show an aberrant development of the root system characterized by swelling (galls or knots), which inhibit the uptake of water and nutrients and interfere with the translocation of minerals and photosynthetic products (Berlinger, 1986). As a result, typical deficiency symptoms appear, such as wilting, stunted growth and early senescence, resulting in severe crop losses. Although chemical control of nematodes is feasible, the adverse effects of nematicides have made breeding for nematode resistant tomatoes essential (Medina-Filho and Tanksley, 1983).

Resistance to root knot nematodes was found to be present in the wild species *L. peruvianum* (Bailey, 1941), and resistant *L. esculentum* cultivars were obtained by introgressing the *L. peruvianum* resistant gene, designated *Mi*, into *L. esculentum* (Frazier and Dennet, 1949). From the initial *L. esculentum x L peruvianum* cross, one single F1 plant was used for further breeding by repeated backcrossing. Presently, all known nematode resistant *L. esculentum* cultivars, which possess this single dominant *Mi* gene are derived from this F1 plant. The *Mi* gene, which confers resistance to all major *Meloidogyne* spp. known to attack tomato, is located at position 44 on chromosome 6 (Koornneef and Zabel, 1990) and is very closely

linked to the leaf colour marker *yv* (yellow virescent) and to the acid phosphatase-1 (*Aps-1*) locus (Medino-Filho, 1980).

Acid phosphatases (APS) belong to a broad group of enzymes that catalyse the hydrolysis of inorganic phosphate from phospho-monoesters at low pH. APS is ubiquitous in nature and activity has been reported in a wide range of plants (Ching *et al.*, 1987; Baker and Takeo, 1973). Plant APS's vary substantially in molecular size, tissue and subcellular location and regulation of expression (Goldstein *et al.*, 1988). Many roles have been postulated for these enzymes in plants, including a role in the release of inorganic phosphate from organic phosphate in the environment under conditions of phosphate limitation (Goldstein *et al.*, 1988). There are several APS isozymes (Aps-1 and Aps-2 isozymes) in tomato that are distinguished by their electrophoretic mobility and/or other properties (Rick and Fobes, 1974). Activity of the Aps-1 isozymes is relatively high in leaflets, hypocotyl tissue and the apical meristem of young plants, but is absent in dry and germinating seed, cotyledons, leaf petioles, petals, anthers and mature fruits (Medino-Filho, 1980). Activity of Aps-1 increases in root tips of both resistant and susceptible tomato plants after exposure to root knot nematodes (Williamson and Colwell, 1991). The *Aps-1* allele, *Aps-1*^{1}, derived from *L. peruvianum*, which was carried along with the *Mi* gene in the original cross, is easily distinguishable from the *L. esculentum Aps-1* alleles, *Aps-1*$^{+}$ and *Aps-1*^{3}, by electrophoresis and isozyme staining (Rick and Fobes, 1974). The fortuitous linkage of the *Mi* locus to the *Aps-1* locus has been exploited in evaluating tomato breeding lines for resistance to root knot nematodes, as the presence of the *L. peruvianum Aps-1*^{1} allele in tomatoes always has been indicative of resistance to nematode infection.

This study was, therefore, conducted (a) to define the plant organs (seeds, mature and immature leaves) in which the *Aps-1*^{1} allele is expressed, (b) to determine *Aps-1*^{1} expression in infested and non-infested plants, (c) to determine if the growth stage of seedlings has an effect on the expression of the *Aps-1*^{1} allele and (d) to confirm if the linkage between the nematode resistant gene (*Mi* gene) and the *Aps-1*^{1} gene could be used for evaluation of breeding lines in the tomato breeding programme at the Vegetable and Ornamental Plant Institute, Pretoria. Most breeding programs used starch gel electrophoresis for this purpose, due to it being less expensive and less toxic (Cap and Roberts, 1992). In this study polyacrylamide gel electrophoresis, with better band resolution, was used for the separation of APS isozymes.

5.2 Materials and methods

5.2.1 Plant material

Seventeen cultivars of *Lycopersicon esculentum*, *L. peruvianum* and four F1 hybrids of *L. esculentum* (Table 5.1) were selected for this study based on their tolerance or susceptibility to infestation from previous greenhouse trials, or since they were bred from *L. peruvianum.* (Pedigrees in Fig. 3.4). The four F1 hybrids originated from crosses made between AceVFN x Rotam-3, AceVFN x Rodade, Karino x AceVFN, Rotam-3 x AceVFN. They were included in the study to determine if the isozyme band which is identified as the *Aps-1*^{1} allele is inherited as a single dominant gene.

5.2.2 Greenhouse evaluation of nematode infestation

Infested soil containing all four *Meloidogyne* spp. (*M. hapla, M. incognita, M. javanica* and *M. arenaria*) were obtained from a source maintained in the greenhouse. The root knot nematode source was established from root cuttings of infested green beans.

Seeds from plants listed in Table 5.1 were sown in seed trays in the greenhouse (30 °C) in infested and non-infested soil. They were evaluated for nematode infestation at the 8th week after germination. The roots were washed and scanned in water, against light, for possible nematode infestation. Susceptible cultivars were identified by bead-like nodes on their roots (nodes were absent in tolerant cultivars) and were scored on a 0 - 100 % scale. Plants were classified into four groups, namely 0 - 25 %, 25 - 50 %, 50 - 75 % and 75 - 100 % infestation.

5.2.3 Acid phosphatase evaluation

Seeds from plants listed in Table 5.1 were sown in seed trays in the greenhouse (30 °C) in infested as well as in non-infested soil. Plants were analyzed for APS activity once a week for 4 weeks and again on week eight after germination. This was done to determine if growth stage of the plant or infestation with nematodes had any effect on the expression of the *Aps-1*^{1} allele which is proposed to be genetically linked to the nematode resistant gene (*Mi*-gene).

F1-hybrids, grown in the greenhouse in infested soil, were analyzed for APS activity only during week 4 after germination.

5.2.4 Isozyme extraction

Crude extracts were made of mature leaves, immature leaves as well as of seeds. Twenty milligram of material was crushed in liquid nitrogen with a mortar and pestle, whereafter 200 µl of extraction buffer (10 ml of 0.05 M Tris-HCl, pH 6.8; 2 ml of glycerol and 100 µl ß-mercaptoethanol) was added (Cap and Roberts, 1992). The crude extract was centrifuged at 10 000 x g for 5 minutes at 4 °C and 25 µl of the supernatant was loaded onto the gel.

5.2.5 Gel preparation

The method of Laemmli (1970) was used except that SDS was excluded from the gel solution. The separating gel (12.75 %) consisted of 4.25 ml of 30 %:0.8 % acrylamide:N,N'-methylene bisacrylamide; 4.25 ml of 0.75 M Tris-HCl pH 8.8; 1.0 ml of H_2O; 0.5 ml of 10 % ammonium persulphate and 3.0 µl TEMED. The 6.0 % stacking gel consisted of 0.75 ml of 30 %:0.8 % acrylamide:N,N'-methylene bisacrylamide; 2.5 ml of 0.25 M Tris-HCl pH 6.8; 0.05 ml of H_2O; 0.25 ml of 10 % ammonium persulphate and 1.5 µl TEMED. The separating gel was left overnight at room temperature to polymerize before casting of the stacking gel. A Bio-Rad mini-PROTEAN II electrophoresis apparatus with a 80 x 100 x 0.75 mm gel format was used.

The running buffer contained 1.4 % glycine and 0.75 M Tris-HCl pH 8.8. Electrophoresis was performed at room temperature at 60 V until the tracking dye (bromophenol blue) reached the bottom of the gel.

The staining method for APS isozymes was modified from that of Vallejos (1983). Fast Black K salt (50 mg); 1 M $MgCl_2$ (0.5 ml) and ß-naphthyl phosphate (15 mg dissolved in 1.5 ml 50 % acetone) was added to 50 ml of a 50 mM sodium acetate buffer pH 5.4 and the gels were stained at room temperature in the dark until the isozyme bands became visible.

5.3 Results

5.3.1 Greenhouse evaluation of nematode resistance

Tolerance or susceptibility of cultivars, *L. peruvianum* and F1 hybrids to nematode infestation in the greenhouse is shown in Table 5.1. Susceptibility or tolerance of plants was estimated in comparison to Rodade (susceptible), the positive and Rotam-4 (tolerant) the negative control (Figure 5.1).

Table 5.1. Cultivars and their susceptibility to nematode infestation.

CULTIVAR	NUMBER OF PLANTS EVALUATED	0 %[1]	0-25%[1]	25-50%[1]	50-75%[1]	75-100%[1]
1. *L. peruvianum*	5	5	0	0	0	0
2. Rodade	9	0	0	0	2	7
3. Rotam-4	8	4	3	1	0	0
4. Rotam-1	24	17	5	2	0	0
5. Stevens	24	10	3	5	6	0
6. M88/44	13	6	6	1	0	0
7. Rossol VFN	21	18	2	1	0	0
8. Hawaii	33	6	6	7	9	5
9. M88/68	15	2	2	0	4	7
10. Piersol	17	17	0	0	0	0
11. AceVFN	15	14	1	0	0	0
12. L1051	21	3	6	8	4	0
13. Rotam-3	24	6	12	4	0	2
14. Flora-Dade	23	11	8	4	0	0
15. Karino	22	1	8	8	2	3
16. Corde'oc	12	2	5	3	2	0
17. Rdpl Albesto	4	4	0	0	0	0
18. BW2	24	8	8	3	3	2
AceVFNxRodade (F1)	5	5	0	0	0	0
AceVFNxRotam-3 (F1)	5	4	1	0	0	0
KarinoxAceVFN (F1)	5	5	0	0	0	0
Rotam3xAceVFN (F1)	6	5	1	0	0	0

[1] percentage infestation

The tomato roots shown on the right hand side in Figure 5.1 is considered to be 100 % infested with nematodes while Rotam-4 on the left hand side had no infestation. Plants were classified into the other three categories, namely 0 - 25 %, 25 - 50 % and 50 - 75 % infestation accordingly.

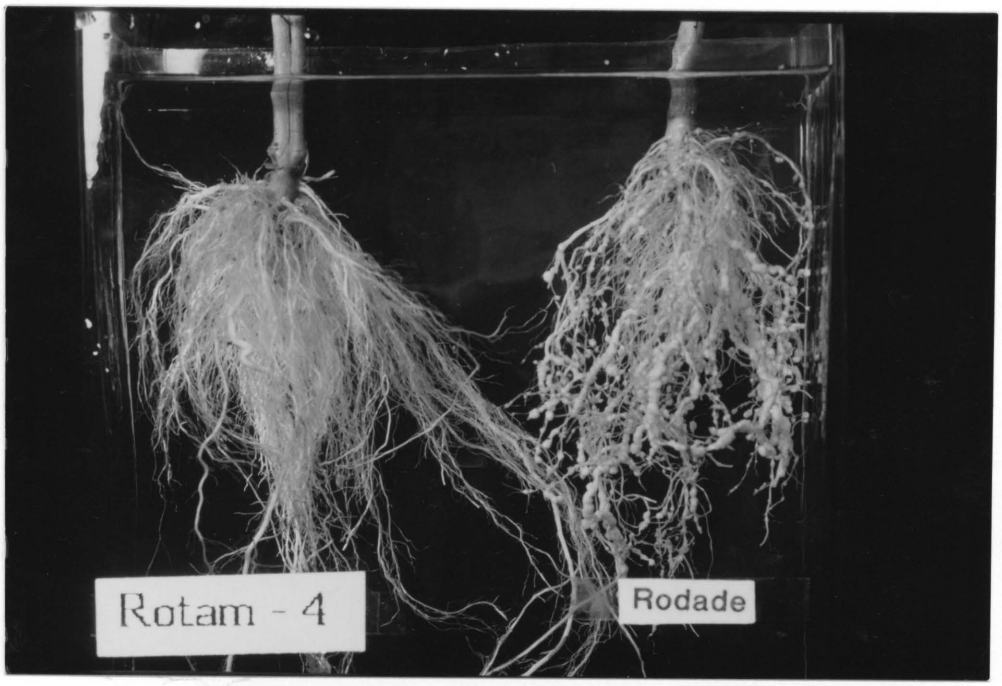


Figure 5.1. Nematode infestation of tomato roots. Rodade on the right is the positive control and Rotam-4 on the left hand side is the negative control.

5.3.2 Acid phosphatase expression in leaves and seeds.

The expression of Aps-1 and Aps-2 in immature, mature leaves and tomato seeds are compared in Figure 5.2. Aps-2 and Aps-1 isozymes are indicated on the photograph. Lanes 1 and 2 show APS isozymes in immature and mature leaves of Rotam-4, respectively. Lanes 3 and 4 show APS isozymes in immature and mature leaves of Rodade, respectively, lane 5

APS isozymes in mature leaves of *L. peruvianum* and lane 6 indicates APS in seeds of Rotam-4. No differences in the number of APS isozymes were detected in lanes 1 - 4, but an increase in isozyme band intensity was noticed in immature leaves of both Rotam-4 and Rodade (lanes 1 and 3). *L. peruvianum* (used as standard) expressed an additional Aps-1 isozyme (closed arrow head), while the Aps-1 isozyme with the highest mobility was absent. Aps-1 isozymes and the Aps-2 isozyme with the highest mobility were not expressed in seeds (lane 6).

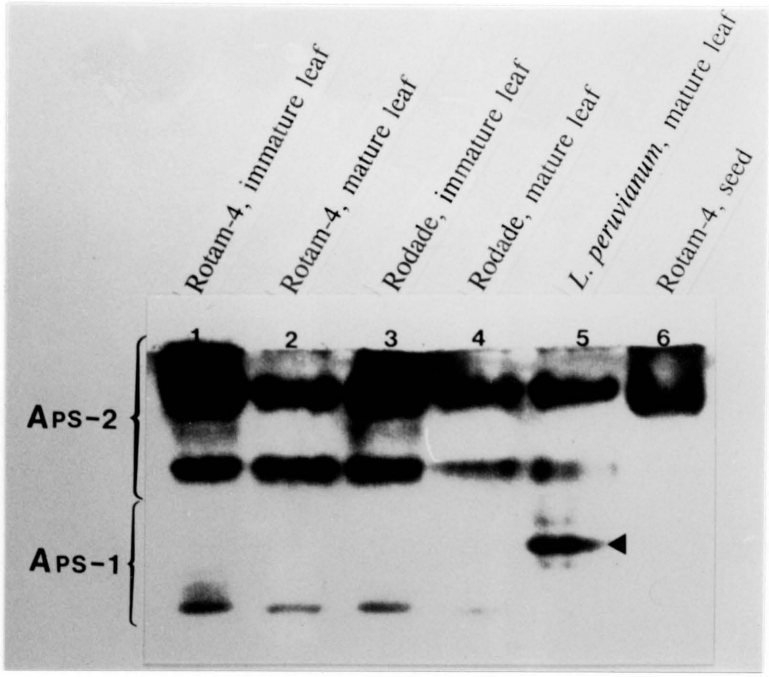


Figure 5.2. Aps-1 and Aps-2 isozymes detected in leaves and seeds after staining of a 10 % PAGE gel. Lane 1 - immature leaf of Rotam-4, lane 2 - mature leaf of Rotam-4, lane 3 - immature leaf of Rodade, lane 4 - mature leaf of Rodade, lane 5 -mature leaf of *L. peruvianum*, lane 6 - seeds of Rotam-4.

5.3.3 Acid phosphatase expression in non-infested and infested plants.

The expression of APS isozymes of seventeen *L. esculentum* cultivars and *L. peruvianum* (Table 5.1), not infested with root knot nematodes, are shown in Figure 5.3 a - e, lanes 1 - 18. Figure 5.3 (a) represents APS isozyme expression during the first week after germination, Figure 5.3 (b) during the second week after germination, Fig. 5.3 (c) the third week after germination, Fig. 5.3 (d) the fourth week after germination and Fig. 5.3 (e) the eighth week after germination. Aps-2 isozymes are indicated by a bracket, and the Aps-1 alleles, *Aps-1*^{1}, *Aps-1*$^{+}$, *Aps-1*^{3} and *Aps-1*^{4}, are indicated as such.

Two Aps-2 isozymes were expressed in all *L. esculentum* cultivars (Lanes 2 - 18) and in *L. peruvianum* (lane 1) throughout eight weeks. The relative band intensity of the two isozymes did not vary visibly over this period. The Aps-2 isozyme in *L. peruvianum* marked with a closed arrow in Figure 5.3 (a) had a slightly higher mobility as than Aps-2 isozymes (open arrow) of *L. esculentum* cultivars, except during the second week after germination when the band mobility became similar to that of the *L. esculentum* cultivars. This difference suggests that a different Aps-2 isozyme was expressed in *L. peruvianum*, except during the second week after germination when the same Aps-2 isozyme as *L. esculentum* was expressed.

L. peruvianum expressed the linked *Aps-1*^{1} allele throughout the period of sample taking, but did not possess alleles *Aps-1*$^{+}$ or *Aps-1*^{3} which were only detected in *L. esculentum* cultivars. Another Aps-1 allele that was not reported before, was noted in *L. peruvianum* during the second week after germination, designated *Aps-1*^{4} (Fig. 5.3 b; closed arrow). This novel allele was expressed during the same period as the distinct Aps-2 isozyme of *L. peruvianum* (Fig

5.3 b). Another Aps-1 allele that was only expressed during the second week was the *Aps-1*^{3} allele of *L. esculentum* cultivars (Fig. 5.3 b; open arrow).

The *Aps-1*$^{+}$ allele was expressed in *L. esculentum* during the whole period of sample taking. The *Aps-1*^{1} allele, linked to the *Mi* gene, was constitutively expressed by only one *L. esculentum* cultivar, namely AceVFN (lane 11). AceVFN never expressed the *Aps-1*$^{+}$ or *Aps-1*^{3} alleles of other *L. esculentum* cultivars and the Aps-1^{4} isozyme that was noted during the second week after germination in *L. peruvianum* was also never expressed by AceVFN.

Exactly the same pattern of Aps-1 and Aps-2 isozyme expression were observed for *L. peruvianum* and *L. esculentum* cultivars after infestation by root knot nematodes (results not shown). The expression of APS isozymes in leaves is thus not induced by nematode infestation of tomato plants.

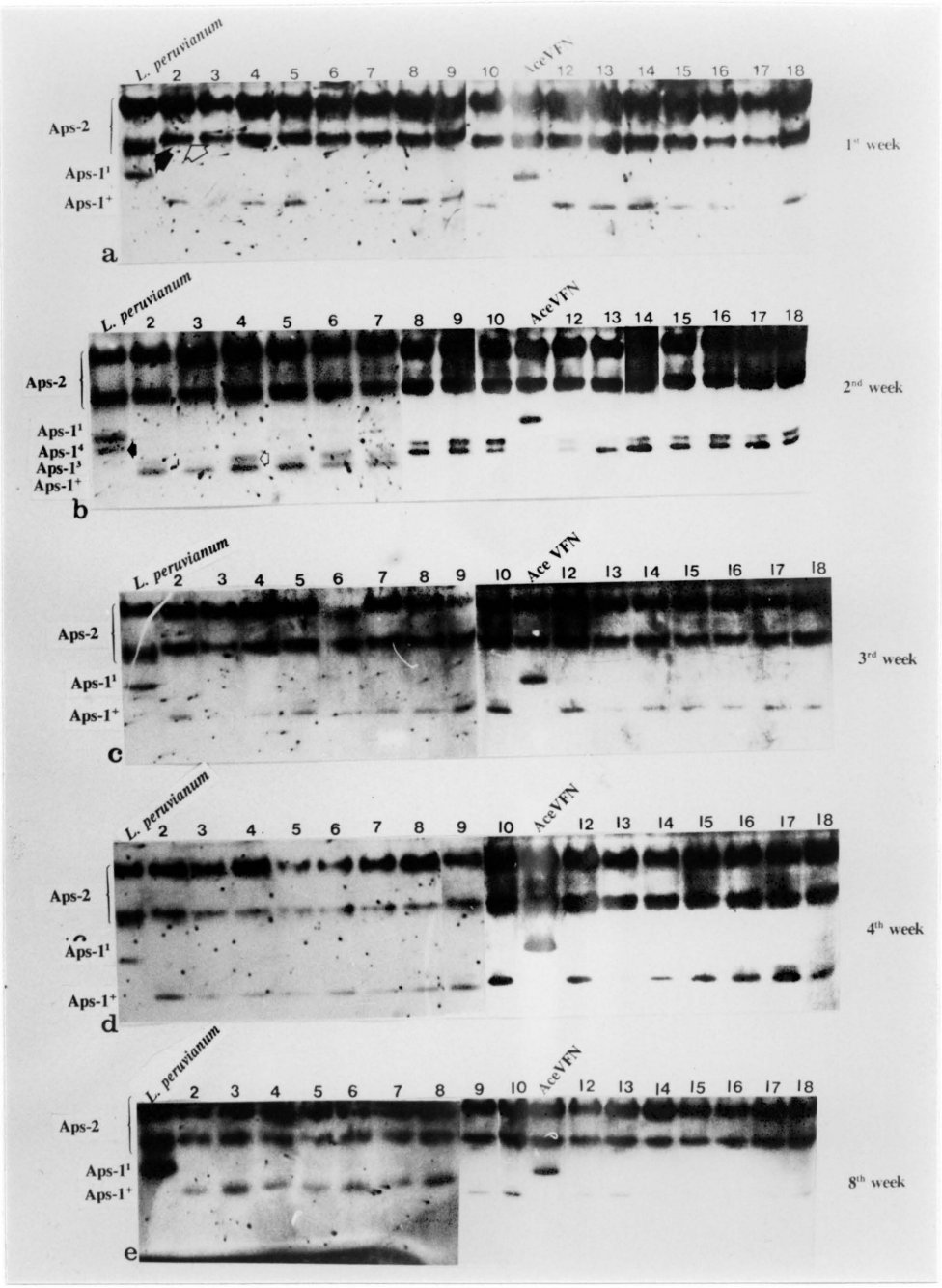


Figure 5.3 a - e. Acid phosphatase isozyme analysis of non-infested *L. peruvianum* (lane 1) and *L. esculentum* cultivars (lanes 2 - 18; Table 5.1) by means of 10 % PAGE. (a) APS isozyme analyses of immature leaves after one week after germination, (b) two weeks after germination, (c) three weeks after germination, (d) four weeks after germination and (e) eight weeks after germination. Aps-2 isozymes are indicated by a bracket and alleles *Aps-1*^{1}, *Aps-1*$^{+}$, *APS-1*^{3} and *Aps-1*^{4} are indicated on the photograph as such. *L. peruvianum* and AceVFN are indicated on the photograph since they are distinct from all the other cultivars.

5.3.4 Acid phosphatase expression in F1 hybrids.

Figure 5.4 shows the expression of APS isozymes in F1 hybrids (Rotam-3 x AceVFN, AceVFN x Rotam-3, AceVFN x Rodade and Karino x AceVFN (Table 5.1), analyzed four weeks after germination.

The two Aps-2 isozymes which were consistently present in all *L. esculentum* cultivars as well as *L. peruvianum*, were also present in each one of the F1 hybrids. A third Aps-2 isozyme with a mobility slightly higher than that of the second Aps-2 isozyme was also observed (open arrow).

The *Aps-1*^{1} allele which was only present in the AceVFN parent (Fig. 5.3), was inherited by all F1 hybrids. The *Aps-1*$^{+}$ allele which was present in all *L. esculentum* cultivars tested (Fig. 5.3) was also inherited by the F1 hybrids. An additional isozyme (Fig. 5.4; closed arrow) with a mobility and band intensity intermediate to the two other Aps-1 isozymes (Aps-1^{1} and Aps-1^{+}) was also apparent. The *Aps-1*^{3} allele was not expressed in the F1 crossings.

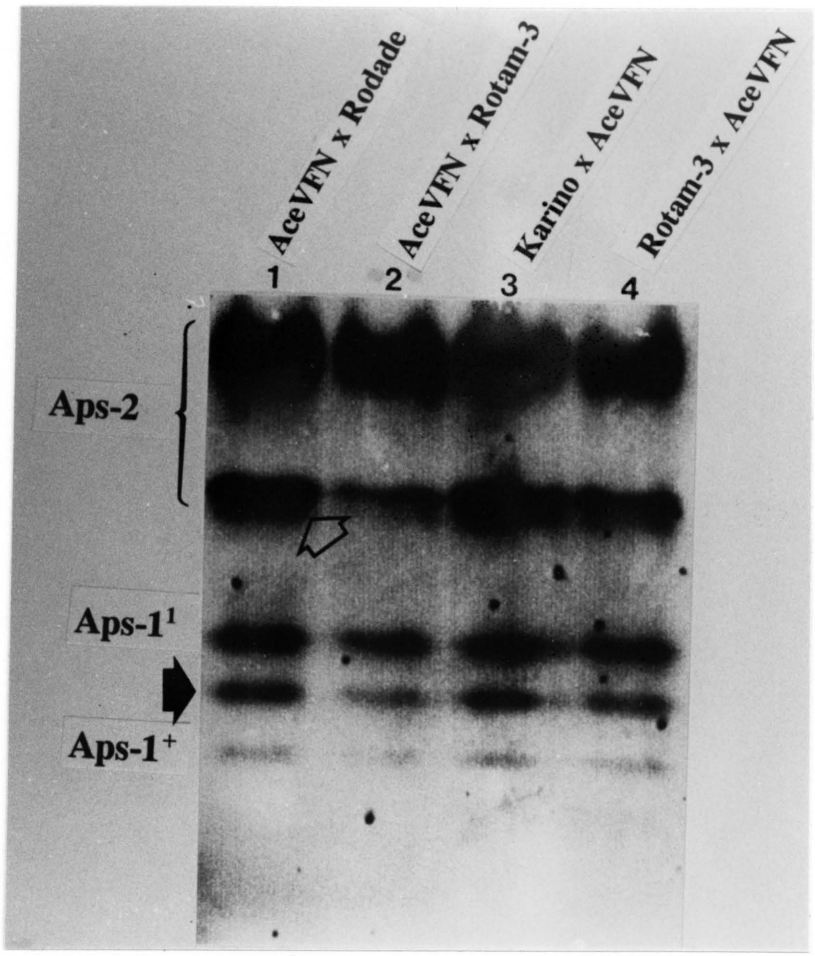


Figure 5.4. Acid phosphatase isozyme analysis of F1 hybrids by means of 10 % PAGE. Lane 1 - AceVFN x Rodade, lane 2 - AceVFN x Rotam-3, lane 3 - Karino x AceVFN, lane 4 - Rotam3 x AceVFN. Aps-2 isozymes are indicated by a bracket and alleles *Aps-1*^{1} and *Aps-1*$^{+}$ are indicated as such. The newly formed Aps-1 isozyme is indicated by a closed arrow and a novel Aps-2 isozyme is indicated by an open arrow.

5.4 Discussion

Cultivars listed in Table 5.1 can be classified into three groups according to their tolerance or susceptibility to nematode infestation in the greenhouse. Plants that were totally resistant were *L. peruvianum*, AceVFN, Piersol, Roodeplaat Albesto and the four F1-hybrids. Susceptible cultivars were Rodade and Stevens, while the rest of the cultivars showed "intermediate" resistance, i.e. not 100 % tolerant or susceptible. These results were unexpected as Gilbert and McGuire (1956) found that resistance of tomatoes to the *Meloidogyne* spp. is conferred by a single dominant gene. This gene was designated *Mi*, since the original resistance screening was made with *M. incognita*. Studies done by Frazier and Dennett (1949) and Barham and Winstead (1957) confirmed these results. Theoretically, plants bearing a single dominant gene for disease resistance should be fully resistant against the particular pathogen.

An explanation for this discrepancy is that two genes are responsible for resistance to nematodes. Cap *et al.* (1993) proposed that cultivars which are resistant to nematodes at high temperatures contain two dominant genes, (*Mi-2* and *Mi*), with only the *Mi* gene present in cultivars which are resistant at lower temperatures. The heat-stable gene (*Mi-2*) is dominant, responsible for resistance to *Meloidogyne* spp. at high temperatures, segregates independently from the *Mi* gene and is not genetically linked to the *Aps-1*^{1} allele. It was speculated that the heat-stable gene originated from the *L. peruvianum* line P.I. 126443, while the heat-unstable gene originated from the *L. peruvianum* line P.I. 270435, and is allelic to, or the same, as the dominant *Mi* gene. Based on this information and results from this study, it is proposed that cultivars AceVFN, Piersol and Roodeplaat Albesto contained the *Mi-2* gene which is

dominantly inherited by the F1 hybrids (totally resistant). The intermediate resistant cultivars by analogy, should contain only the *Mi* gene which confer partial resistance at the higher temperatures (30 °C) employed in this study. Susceptible cultivars contained the recessive *mi* gene.

L. peruvianum, AceVFN and the four F1 hybrids were the only resistant plants that expressed the *Aps-1*^{1} allele (Fig's. 5.3 and 5.4). Cultivars Piersol and Roodeplaat Albesto were also resistant to nematodes at the higher temperature (Table 5.1), but did not express the *Aps-1*^{1} allele. The intermediately resistant cultivars as well as the susceptible cultivars also did not express this isozyme. Based on the discussion above it is postulated that *L. peruvianum* and AceVFN contain both the *Mi-2* and *Mi* genes, since both plants displayed resistance at elevated temperature (*Mi-2* gene) and also the linked *Aps-1*^{1} allele (genetically linked to the *Mi* gene). The other two resistant cultivars, Piersol and Roodeplaat Albesto, which did not express the *Aps-1*^{1} allele, thus contain the *Mi-2* gene and either lack the *Mi* gene or this gene was unlinked to the *Aps-1*^{1} allele. The second probability is supported by results of classical genetic methods which showed that cultivars which are resistant at high temperatures, possess both nematode resistant genes (Cap *et al.* 1993). The cultivars displaying intermediate resistance most likely contain only the *Mi* gene, but the *Aps-1*^{1} linkage was lost due to a crossover between the *Aps-1*^{1} and *Mi* loci in the early generations of these cultivars (Klein-Lankhorst *et al.*, 1991a). The susceptible cultivars, Rodade and Stevens, then possess the recessive *mi* gene which is not linked to the *Aps-1*^{1} allele (Cap *et al.*, 1993). The four F1 hybrids inherited both the *Mi* and *Mi-2* genes as single dominant, due to the fact that they were nematode resistant at high temperatures and that the linked *Aps-1*^{1} allele were also expressed (Fig. 5.4).

All *L. esculentum* cultivars expressed the *Aps-1*$^{+}$ allele, except for AceVFN and *L. peruvianum* which both expressed the *Aps-1*^{1} allele (Figure 5.3). The *Aps-1*$^{+}$ allele was constitutively expressed from week 1 until week 4 after germination and also at week 8. The existence of the *Aps-1*^{3} allele was only recently discovered by Klein-Lankhorst, *et al.* (1991a) and our data show that this isozyme was exclusively expressed in *L. esculentum* cultivars during the second week after germination (except AceVFN; Fig 5.3 b). *L. peruvianum* expressed a novel isozyme, named Aps-1^{4}, that was only observed during the second week after germination. The *Aps-1*^{4} and *Aps-1*^{3} isozymes, therefore, were developmentally regulated, dependent on seedling age.

The Aps-2 isozymes of *L. esculentum* cultivars and of *L. peruvianum* (Fig. 5.3) were constitutively expressed throughout the whole period of sample taking, but during the second week after germination a new *L. peruvianum* isozyme appeared, which had the same mobility as that of the *L. esculentum* Aps-2 isozyme (Figure 5.3). The isozymes (exclusively expressed during the second week) Aps-1^{3}, Aps-1^{4} and Aps-2 of *L. peruvianum* probably function during the forming of the first true leaves of the tomato plants which appeared during this period.

The F1 hybrids (AceVFN x Rodade; AceVFN x Rotam-3; Karino x AceVFN; Rotam-3 x AceVFN) expressed the *Aps-1*^{1}, *Aps-1*$^{+}$ and *Aps-2* alleles, but not the *Aps-1*^{3} allele of *L. esculentum* or the *Aps-1*^{4} allele of *L. peruvianum* (Fig. 5.4). The Aps-1^{1} isozyme, therefore was inherited from parent AceVFN (Fig. 5.3) and the Aps-1^{+} isozyme from the parents Rodade, Rotam-3 and Karino (Fig. 5.3). Both the *Aps-1*^{1} and *Aps-1*$^{+}$ genes were, therefore, inherited in a dominant manner, while the absence of the *Aps-1*^{3} and *Aps-1*^{4} alleles suggested a recessive nature. The inheritance of Aps-1^{1} isozyme was not influenced by the gender of

AceVFN (male or female parent), accordingly the conclusion can be drawn that the *Aps-1*^{1} allele is carried in the genome and not as part of the mitochondria or chloroplast DNA. Another Aps-1 isozyme with intermediate mobility and intensity equal to the Aps-1^{1} and Aps-1^{+} isozymes was present on the gel. This implies that both Aps-1^{1} and Aps-1^{+} are probably homodimeric isozymes and that monomers may reorganise during posttranslation to form a heterodimer (arrow in Fig. 5.4).

Tomato seeds did not express Aps-1 isozymes and only one Aps-2 isozyme, while leaves expressed one Aps-1 and two Aps-2 isozymes (Fig. 5.2). Identical Aps-1 and Aps-2 isozymes are expressed in both mature and immature leaves, but band intensities were slightly different between the Aps-2 isozymes of mature and immature leaves. It can thus be concluded that the age of a leaf does not influence the expression of APS isozymes. Leaves of seedlings can, therefore, be screened for nematode resistance at a young age which saves time when breeding lines are evaluated.

To summarize, results in this study suggest that nematode resistant tomato cultivars can be classified into two groups, namely those containing the *Mi* gene which contributed to resistance at lower temperatures and those containing both the *Mi-2* and *Mi* genes responsible for resistance to nematodes at high temperatures (± 30 °C). The *Aps-1*^{1} allele is linked to the *Mi* gene (not to the *Mi-2* gene), but not all tomato cultivars having the *Mi* gene, express the Aps-1^{1} isozyme, probably due to a crossover which occurred in early generations. Therefore, screening of tomato breeding lines for the Aps-1^{1} isozyme is only feasible for cultivars in which this linkage is intact. Cultivars containing the *Mi-2* gene, unfortunately is not selectable by this method. From results obtained in this study, only AceVFN acquired the *Aps-1*^{1} - *Mi*

linkage, which make the application of this method in a breeding program only partly effective. However, RFLP probes closely linked to the *Mi* gene do exist and evaluation of nematode resistance by means of RFLP probes would be the appropriate method under these circumstances. RAPD's as molecular markers for root knot nematode resistance in tomatoes are at present being investigated (Klein-Lankhorst *et al.*, 1991b) and promise to be an easy and cheaper way to evaluate breeding lines.

CHAPTER 6

6.1 CONCLUDING DISCUSSION

6.1.1 Cultivar identification

Newly bred cultivars should possess a unique combination of genes which differ from every other cultivar of the same crop. It is essential for plant breeders to identify unique characteristics of a new cultivar. Traditionally, novel cultivars were only characterized by morphological properties, such as fruit mass, fruit size, shape of the fruit, growth habit, fruit colour and days to maturity, etc. These features are not reliable as it is influenced by environmental conditions, such as temperature, rain, hail or fertilization. Newly bred cultivars are distinguished by physiological traits, such as disease resistance, but environmental conditions could disguise these qualities (Bailey, 1983).

In the case of tomatoes, the self-pollinating nature and breeding method used contribute to the narrow gene pool found within cultivars (Miller and Tanksley, 1990a). Only one initial cross between two parents are made and breeding lines are visually selected from the second generation (F2) for desirable phenotypes. From the fourth generation, breeding lines are also selected for disease resistances until the F10 generation when breeding lines are accepted as purely bred. This kind of breeding line selection results in little or no morphological differences between cultivars and breeders need more effective methods for distinguishing between them. Biochemical methods generally used for cultivar identification are protein analysis, isozyme analysis and DNA based methods, such as RFLP's and RAPD's (Rafalski

et al. 1991). No standard cultivar identification method for tomatoes is available and this study was initiated to investigate potential methods for cultivar identification. Protein separating techniques, such as SDS-PAGE, gradient-PAGE and IEF on leaves and seeds (Figures 2.1 to 2.4) were performed and protein profiles were compared. None of these methods, however, were able to discriminate between any one of the cultivars studied.

An alternative method for cultivar identification is isozyme analysis, since it is easy to perform, cheap and can be automated for routine use. Six isozyme systems were tested, namely ADH, EST, APS, PGI, PGM and 6-PGDH. Since the expression of esterase isozymes were found to be dependent on the developmental stage of tomato leaves (Figure 3.2), seeds were used for further studies. Isozyme profiles of dry seeds, obtained by isoelectric focusing, were highly reproducible (Fig. 3.3). IEF as electrophoretic method and acrylamide as separating medium proved to be more successful in separating isozymes than starch gel electrophoresis (Rick and Yoder, 1988). Twelve of the seventeen cultivars, used in this study could be distinguished (Table 3.2). However, isozyme analysis could not discriminate between closely related cultivars (common breeding parents), such as the cultivars bred at Roodeplaat. Further investigations using other isozyme systems need to be undertaken to fully evaluate the potential of this method.

Additional studies were directed to more discriminatory methods, such as DNA based methods. The two DNA based methods most frequently used for identifying cultivars are, RFLP's and RAPD's (Parent and Page, 1992; Wilkie *et al.* 1993). RAPD's are cheaper and less laborious to perform than RFLP's, which makes it the preferential method to develop for identification of tomato cultivars (Ragot and Hoisington, 1993). RAPD's has the advantage

that the whole genome is screened for potential polymorphism between the DNA of two individuals with random 10-mer primers (Williams *et al.* 1990). Closely related cultivars and breeding lines, were used to test the discriminating power of the RAPD method. Reproducible results were obtained under optimised conditions, but none of the five primers used in this study were able to discriminate between the genomes of the tomato plants (Fig. 4.5). Obviously, five random primers are insufficient in number to allow a final conclusion and it is suggested that more primers should be tested. However, a disadvantage of a method like RAPD's, which screen the whole genome, is that amplified non-coding DNA could be mistakenly regarded as a genetic difference. RFLP's was not investigated in this study due to the unavailability of appropriate probes. However, this method should be considered for further investigations as Broun *et al.* (1992) reported that two RFLP probes, which they employed, were able to distinguish between some tomato cultivars.

Although the genetic diversity observed between tomato cultivars by means of isozyme analysis was a major breakthrough, isoelectric focusing of seed isozymes was not capable of discriminating between closely related tomato cultivars, advanced breeding lines and their progeny. Therefore the challenge still remains to find an appropriate biochemical method for discriminating effectively and reliably between tomato cultivars.

6.1.2 Disease resistance evaluation

As stated in chapter 5, the selection of breeding lines for disease resistance starts during the fourth generation. Traditionally, breeding lines grown in a greenhouse, are inoculated with the pathogen and classified as susceptible, or tolerant depending, respectively, on the appearance

or absence of typical disease symptoms. This selection method is expensive, labour intensive and unreliable as environmental conditions, such as temperature and photoperiod, play a role in the expression of the disease symptoms (personal communication, tomato breeders). An expert pathologist is also needed to identify the symptoms since it could be mistaken for nutrient deficiencies. Advanced breeding lines are evaluated for disease resistances in field trials, which makes it even more expensive and labour intensive, eg. infested fields need to be isolated from other fields to prevent cross contamination, farming equipment need to be sterilized, etc. However, these difficulties can be avoided if breeding lines are evaluated by means of molecular methods. Molecular methods commonly used to evaluate tomato breeding lines for disease resistances are isozymes, RFLP's, and recently also RAPD's (Bournival *et al.*, 1989; Burr *et al.*, 1983; Martin *et al.*, 1991).

An investigation was undertaken of the genetic linkage between the root knot nematode resistant allele (*Mi*) and the isozyme expressed by the *Aps-1*^{1} allele (Medino-Filho, 1980). The aim was to evaluate the feasibility of a routine molecular technique which could select tomato breeding lines for root knot nematode resistance reliably and at an early stage of plant development and at low costs. An electrophoresis technique was employed for the efficient separation of Aps-1 and Aps-2 isozymes from leaves. The expression of APS isozymes in leaf tissue in contrast to EST isozymes (Fig. 3.2), did not vary over time, except for the expression of a number of new isozymes only during the second week after germination.

Cultivars were first evaluated in the greenhouse for nematode resistance. Contrary to what was expected from a single dominant gene, some of the plants exhibited intermediate resistance, while others were totally resistant or susceptible to infestation. Cap *et al.* (1993)

used classical genetic studies to prove that plants which are resistant at high temperatures (30 °C) contained the *Mi* (heat-unstable) as well as *Mi-2* (heat-stable) alleles. Since this study was performed at 30 °C, the conclusion was drawn that two kinds of nematode resistant genes existed, one effective at high temperatures and another one which is only partly effective at high temperatures, but responsible for resistance at lower temperatures (25 °C). Consequently, it is suggested that plants resistant at lower temperatures, only contained the *Mi* allele while susceptible cultivars possess the recessive *mi* allele. However, conclusive support should be obtained by comparing the susceptibility or tolerance of the same plants to nematode infestation at 25 °C and 30 °C. If confirmed, the practical implication will be that breeding lines should be selected at elevated (30 °C) greenhouse temperatures to be suitable for the summer season in South Africa.

The same cultivars were also evaluated for the expression of the *Aps-1*^{1} allele. Since this allele is genetically linked to the *Mi* allele, plants with the Aps-1^{1} isozyme should be resistant to root knot nematodes. Although literature reports state a definite genetic linkage between the *Aps-1*^{1} and the *Mi* alleles (Gilbert and McGuire 1956), not all resistant cultivars employed in this study exhibited the Aps-1^{1} isozyme (Fig. 5.3). Cap and Roberts (1992) found the same phenomenon and speculated on a possible overcrossing that could have occurred between these two alleles in earlier generations.

During this investigation, a novel Aps-1 isozyme was observed for *L. peruvianum* (Aps-1^{4}) which was co-expressed with the Aps-1^{3} isozyme of *L. esculentum* cultivars during the second week after germination (Fig. 5.3). A developmental function for these isozymes is implied as the first true leaves appeared during this period.

Cultivars used in the tomato breeding program at Roodeplaat, do not exibit the linkage between the *Mi* and *Aps-1*^{1} alleles and selection based on the presence or absence of the Aps-1^{1} isozyme is of limited value. However, RFLP probes for the *Mi* gene recently become available (Klein-Lankhorst *et al.* 1991a), but its usefulness as selectable marker in the breeding programme at the Vegetable and Ornamental Plant Institute, Pretoria needs to be established. However, the RFLP protocol is extensive and not easily automated, therefore it is suggested that a RAPD marker for nematode resistance should also be considered.

Two important conclusions can be drawn from results of these studies. Firstly, a distinction should be made between tomato breeding lines selected for root knot nematode resistance for regions with high summer temperatures and those selected for regions with moderate summer temperatures. Secondly, the general assumption that the gene responsible for nematode resistance for tomatoes is a single dominant gene and necessarily linked to the *Aps-1*^{1} allele, should be reevaluated.

6.2 SUMMARY

The novelty of newly bred cultivars need to be established, since a certificate of protection is granted to breeders to protect their plant breeders' rights. Traditionally, newly bred cultivars were typified by means of unique morphological traits. Tomato cultivars, however, are morphological very similar due to breeding methods used. Such cultivars lack morphological differences and more sensitive methods are therefore needed to define novelty. Biochemical methods, such as protein and isozyme analysis and DNA based methods were investigated as alternative methods in the attempt to identify genetically uniform tomato cultivars.

Protein separating methods used included SDS-PAGE of seed and leaf polypeptides, isoelectric focusing (IEF) and gradient-PAGE of leaf proteins. None of these methods, however could distinguish between the cultivars and six isozyme systems were evaluated as an alternative method. Seed isozymes alcohol dehydrogenase, acid phosphatase, phosphogluco mutase, phosphogluco isomerase, esterase and 6-phosphogluconate dehydrogenase (ADH, APS, PGM, PGI, EST and 6-PGDH, respectively) separated by means of vertical isoelectric focusing successfully distinguished between 12 of the 17 cultivars used in this study. However, closely related cultivars did not reveal any polymorphism. In an attempt to find differences between them, the study was directed to a DNA based method (RAPD's), which is more sensitive than methods detecting polymorphism at the protein level. $MgCl_2$ and DNA template concentrations were optimized to ensure reproducible banding patterns. The five 10-mer random primers used, displayed different banding patterns, but did not display differences between different cultivars.

Although the genetic diversity observed between tomato cultivars by means of isozyme analysis was a major breakthrough, this study need to be further pursued by using more isozyme systems and/or more random primers and/or restriction fragment length polymorphism to distinguish between closely related tomato cultivars.

The genetic linkage between the *Mi* (single dominant gene for resistance to root knot nematodes) and *Aps-1*^{1} (encoding Aps-1^{1} isozyme) alleles were investigated to assist in identifying root knot nematode resistance in breeding lines. Cultivars were firstly evaluated in the greenhouse at 30 °C for root knot nematode resistance or tolerance. Intermediate, tolerant and susceptable cultivars were identified, which support the postulation by Cap *et al.* (1993) that two genes for nematode resistance exist, namely a gene active at high temperatures (*Mi-2*) and a gene contributing to resistance at lower temperatures (*Mi*).

The same cultivars were also analyzed for the expression of the linked *Aps-1*^{1} allele by means of native-PAGE. The *Aps-1*^{1}, *Mi* linkage were found in some resistant cultivars, while other resistant cultivars as well as the susceptable cultivars did not express the *Aps-1*^{1} allele. It is postulated that an overcrossing took place between the linked *Mi* and *Aps-1*^{1} alleles in earlier generations. This implies that tomato breeding lines whose parents underwent this overcrossing can not be selected for root knot nematode resistance based on the presence or absence of the Aps-1^{1} isozyme.

The novel Aps-1^{4} and Aps-2 isozymes of *L. peruvianum* and the Aps-1^{3} isozyme of *L. esculentum* were co-expressed during the second week after germination which suggested a regulatory function, since the first true leaves of a seedling appear during this period.

Novel information gained from this study are: (a) Tomato cultivars also showed intermediate resistance to nematodes. (b) Isozymes, Aps-1^4 and some Aps-2 isozymes, were detected for the first time. (c) Aps-1^4 and Aps-1^3 isozymes are developmentally regulated. (d) No locally bred cultivars exibited the *Mi/Aps-1*^{1} linkage. (e) Aps-1^+ and Aps-1^1 isozymes are dominantly inherited and (f) are homodimers.

OPSOMMING

Unieke eienskappe van 'n nuutgeteelde kultivar moet bevestig word, sodat 'n sertifikaat ter beskerming van die plantteler se regte uitgereik kan word. Nuutgeteelde kultivars word tradisioneel geïdentifiseer deur middel van unieke morfologiese eienskappe. Tamatiekultivars is egter morfologies soortgelyk weens die aard van die toegepaste teelmetode. Meer sensitiewe metodes is dus nodig vir die bevestiging van 'n nuutgeteelde kultivar se unieke eienskappe. In hierdie studie is biochemiese metodes, soos proteïen- en isosiemanalises sowel as DNA-gebaseerde tegnieke as alternatiewe metodes gebruik in 'n poging om geneties en morfologies identiese tamatiekultivars te identifiseer.

Die proteïen-skeidings metodes wat gebruik is vir moontlike kultivar identifikasie, is SDS-PAGE, vertikale IEF en gradiënt-PAGE van blaar- of saadekstrakte. Nie een van hierdie tegnieke kon onderskei tussen die kultivars nie. Ses saad isosiem-sisteme is ook getoets vir die bepaling van polimorfismes tussen tamatiekultivars. Hulle was alkohol dehidrogenase, suurfosfatase, fosfoglukomutase, fosfogluko-isomerase, esterase en 6-fosfoglukonaat dehidrogenase (ADH, APS, PGM, PGI, EST en 6-PGDH, respektiewelik) en is geskei deur middel van vertikale IEF en 12 van die 17 kultivars kon onderskei word. Kultivars wat

geneties nou verwant is, het egter nie polimorfisme getoon nie. In 'n poging om verskille tussen hulle waar te neem is 'n DNA-gebaseerde metode, naamlik "Random Amplified Polymorphic DNA" (RAPD) getoets. Vyf 10-meer voorvoerders is gebruik en elkeen was verantwoordelik vir die amplifisering van 'n ander deel van die genomiese DNA. Geen inter-kultivar verskille kon egter waargeneem word nie. Hierdie studie behoort voortgesit te word met die gebruik van meer voorvoerders en/of restriksie fragment lengte polimorfisme (RFLP).

Alhoewel die polimorfisme wat tussen kultivars verkry is d.m.v. isosiem analises 'n groot deurbraak was, word daar voorgestel dat nog ensiem sisteme en/of nog RAPD voorvoerders en/of restriksie fragment lengte polimorfisme gebruik moet word om nou verwante kultivars te identifiseer.

Die genetiese koppeling tussen die *Mi* (enkel dominante geen vir bestandheid teen knopwortel nematodes) en *Aps-1*^{1} (kodeer vir die Aps-1^{1} isosiem) allele, is ondersoek sodat dit gebruik kan word as hulp in die evaluasie van teellyne vir nematode bestandheid in 'n tamatieteelprogram. Die gekose kultivars is eerstens in 'n glashuis (30 °C) vir bestandheid of vatbaarheid geëvalueer. Intermediêre, bestande sowel as vatbare plante is verkry, wat die teorie van twee of meer gene betrokke by bestandheid, in plaas van die tradisionele siening van 'n enkel dominante geen, ondersteun. Een alleel is waarskynlik verantwoordelik vir bestandheid by hoë temperature (*Mi-2*) en die ander een by gematigde temperature (*Mi*).

Dieselfde kultivars is ook geanaliseer vir die uitdrukking van die Aps-1^{1} alleel deur middel van natiewe-PAGE. Die *Aps-1*^{1}*/Mi* koppeling het voorgekom by sommige bestande plante, terwyl die meeste bestande plante nie die koppeling gehad het nie. Hierdie verskynsel word

verklaar deur 'n moontlike oorkruising wat plaas gevind het tussen die *Aps-1*^{1} en die *Mi* allele in vorige generasies. Die gevolg van hierdie oorkruising is dat teellyne en ouers wie se voorgeslagte onderworpe was hieraan, nie geëvalueer kan word deur die seleksie van die Aps-1^{1} isosiem nie.

Die nuwe Aps-1^{4} en Aps-2 isosieme van *L. peruvianum* en die Aps-1^{3} isosiem van *L. esculentum* is slegs tydens die tweede week na ontkieming uitgedruk, wat 'n regulatoriese funksie impliseer, aangesien die eerste ware blare van 'n saailing ook tydens hierdie periode verskyn.

Nuwe inligting wat uit hierdie studie voortgespruit het, is die volgende: (a) Tamatiekultivars besit ook intermediêre bestandheid teen nematodes. (b) Aps-1^{4} en sommige Aps-2 isosieme is vir die eerste keer opgemerk. (c) Uitdrukking van isosieme Aps-1^{4} en Aps-1^{3} is afhanklik van die plant se ontwikkelingsstadium. (d) Plaaslike kultivars het nie die *Mi/Aps-1*^{1} koppeling nie. (e) Aps-1^{1} en Aps-1^{+} isosieme word dominant uitgedruk en (f) is homodimeries.

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