

Genetic Stability of Potato (*Solanum tuberosum* L.)
after *in vitro* culture

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The 'not-so-humble' potato

Abstract

In vitro manipulations are being used increasingly for the improvement of potato breeding and cultivation. However, the problem of genetic instability in tissue culture, termed somaclonal variation, has raised the question that *in vitro* manipulation may affect the genetic make-up of the regenerated plants. To answer this question, potato plants have been produced from different *in vitro* regimes including micropropagation *in vitro* storage and regeneration from leaf-disc derived callus tissue.

A method of assessment of stability based on restriction fragment analysis has been developed to analyse these plants. This method has been compared with other methods in which electrophoresis of soluble tuber proteins or analysis of field morphology have been used to assess variability. Although the restriction fragment analysis is more complex, its use can lead to the production of results more quickly than the other methods, which are based on a phenotypic assessment and thus require the growth of the regenerated plants before analysis.

All examples of variation found were among the plants regenerated from leaf-disc callus. This observation supports the view that regeneration from single cells via a callus phase may cause somaclonal variation. The fact that no variants were found in the populations of plants regenerated from intact meristems (i.e. the micropropagation and storage regimes) does not prove that this does not cause somaclonal variation, but the extent of the variation is certainly much lower than that observed in the callus derived population.

These results are discussed within the context of use of *in vitro* manipulations in potato breeding and seed tuber production, especially in developing countries. The requirements for stability and the preferred method for testing, is considered for different applications.

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This thesis is dedicated to my parents

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Abbreviations

°C	degrees Centigrade
Amp	Ampere
BAP	6-Benzylamino purine
Bis	N,N'-methylene bisacrylamide
BSA	Bovine serum albumin
CCC	Chlorocholine chloride
Ci	Curie
cm	centimeters
$\alpha^{32}\text{PdATP}$	5' [alpha ^{32}P] 2'-deoxyadenosine 5'triphosphate
dATP	2'-deoxyadenosine 5'triphosphate
dCTP	2'-deoxycytidine 5'triphosphate
dGTP	2'-deoxyguanosine 5'triphosphate
dTTP	2'-deoxythymidine 5'triphosphate
DMAP	Di-methylaminopropionitrile
DNA	Deoxyribo nucleic acid
dpm	disintegrations per minute
EDTA	Ethylene diamine tetra-acetic acid (disodium salt)
g	gramme
GA ³	Gibberellic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Kb	Kilobase
l	litre
m	metre
ml	millilitre
mg	milligramme
MS	Murashige and Skoog
MW	molecular weight
NAA	Naphthyl acetic acid
ng	nanogramme
OD	Optical density
pg	picogramme
RES	Rothamsted Experimental Station
rpm	revolutions per minute
SCRI	Scottish Crops Research Institute
SDS	Sodium dodecyl sulphate
TCA	Tri-chloroacetic acid
Tris	Tris-Hydroxymethylaminomethane
UV	Ultra-violet
V	Volts
µg	microgramme
µl	microlitre
SDW	Sterile distilled water

Chapter 1. Introduction

1.1 Potato as a world food crop

The potato (*Solanum* spp) is possibly the most underrated food crop in the world. It ranks fourth in world production volume, fifth in dollar value of production, and is near the top of the list for protein and energy production per hectare per day (Horton and Sawyer 1985). It also contains high quality protein and substantial amounts of vitamins, minerals and trace elements (Woolfe 1986). Despite this, most researchers and policy-makers fail to realise its importance and so underestimate its actual and potential role in feeding the world's growing population (Horton and Sawyer 1985). Much of this is probably because of its relatively small contribution to world trade and its image as a low-value staple in most developed countries. Looking more closely at the patterns of cultivation and consumption, especially in developing countries, the importance of the potato crop is revealed and the potential for growth is evident.

1.1.1 The role of potato in world trade

The problems of storage and transport mean that few root and tuber crops figure prominently in world trade. Seed crops (rice, wheat and maize) comprise the major part of world trade in agricultural produce and so have been the major focus of attention for increased use. Breeding and research on these crops is more advanced and very profitable, and has

resulted in significantly increased yields (McMullen 1987). Potato, on the other hand, is a vegetatively propagated crop with mainly a locally based pattern of consumption. This makes it a much less attractive proposition for improvement and so this aspect has been neglected, for example, by companies intent on making profits. Even in Western Europe and North America (where the vast majority of trade takes place and where cultivation is advanced) the introduction of new varieties is slow, with some cultivars being in use for the last 40 years or more (Ross 1985).

1.1.2 Potential for growth in potato production

Total potato production in developed countries is falling slowly, especially in Western Europe. Because of its low-status image as a 'starchy tuber', demand tends to fall as incomes rise and more exotic staples become available. Also, price has little effect on consumption and so, in Britain, production quotas are in place to prevent overproduction. In developing countries, however, the potato has the status of a luxury vegetable and so demand rises as incomes rise and consumption is also price-related (lower price=greater demand). This means that the potential for growth in production is considerable. In fact, in the last two decades, the rate of increase of potato production has been nearly twice that of total food production, and substantially higher than that of most other food crops (Horton and Sawyer 1985). Coupled with the exceptionally high nutritional value mentioned earlier, this makes the potato a very promising candidate for a greater role in

feeding the world's growing population.

1.2 Constraints on potato production in developing countries

The potato evolved as a highland crop grown in subtropical areas, typically rainfed and particularly susceptible to environmental extremes. Highland farmers generally cultivate small plots of land and retain a portion of the harvest to use as seed tubers for the next season. To maintain yields in the high-intensity cultivation of the large lowland areas used for potato production in developed countries, high inputs of fertilisers and pesticides and often irrigation are required. High quality 'certified' (virus-free) seed tubers are purchased each year in order to ensure healthy planting material.

As production expands in developing countries, similar lowland areas - arid or tropical - are being brought into cultivation. The high inputs of developed country agriculture are difficult to reproduce in these countries and while the cultivation is a problem, by far the most important factor is the availability of high quality seed well adapted to the local environment. Use of fresh planting material each year, bred to perform well in the conditions, will improve yields in the traditional highland areas as well, even in the absence of many of the other inputs. The solution to this involves two areas of research; the breeding of new cultivars adapted to local conditions, and the development of seed multiplication systems to produce good quality

virus-free planting material in large amounts.

1.2.1 Production of new cultivars

The potato crop is vulnerable to attack by many pests and diseases and is susceptible to environmental extremes. Although most of these problems are not important in many potato growing areas, they are much greater in developing countries (where, for example, control of disease vectors is more difficult) and thus impose a serious constraint on expansion into new areas. Cultivars well adapted to northern latitudes, that are grown in developed countries, often have low resistance to pathogens and environmental stresses common in hotter climates, and so are of little use in countries with different climates and low-input farming systems. Breeding for resistance and adaptation to new environments offers the best avenue for increased yield in these countries.

The European potato - *Solanum tuberosum* L. - is only one of more than 150 wild and cultivated tuber-bearing species (Hawkes & Hjerting 1969). Thousands of primitive varieties of various *Solanum* species are cultivated in many different environments and represent an enormous germplasm pool. This source of genetic variation is being used increasingly to introduce desirable characters into high-yielding lines, by new *in vitro* techniques as well as conventional breeding programmes (reviewed in Ross 1985). However, the natural diversity is rapidly disappearing as natural habitats are destroyed or brought into cultivation. It is relatively

simple (although quite expensive) to maintain germplasm stocks of seed-bearing crops (Roberts 1989) but crops that reproduce vegetatively cannot always be stored as seed. It is both costly and dangerous for such crops to be maintained in field conditions year after year because valuable lines may easily be lost due to infection or adverse conditions. An *in vitro* germplasm conservation programme solves this problem and many are being set up world-wide for the maintenance of crops with recalcitrant or unsuitable seeds (eg. the International Potato Centre, Lima, Peru).

1.2.2 Seed tuber multiplication systems

The availability of good quality improved seed tubers is also paramount to improved yields. Replanting tubers from the previous harvest leads to cumulative infection with pathogens (especially viruses) and so reduces yields. Seed certification systems, as exist in developed countries, are not practical in many developing countries as the infrastructure for certification, transport and marketing is inefficient or not present at all. The large bulk of planting material required means that transport costs are high, and so production of seed tubers close to areas of use would be ideal. Production of these tubers by multiplication in the field would inevitably lead to infection and loss of yield. A system of micropropagation under sterile conditions would provide rapid multiplication and maintain the pathogen-free status of the plants (this approach is already being investigated in developing countries - Bryan 1988). The link between reduced number of field generations and in-

creased yield is well established in developed countries and is leading to increased transfer of *in vitro* material direct into the field (Jones 1988).

1.3 *In vitro* manipulation of potato

In vitro manipulation of potato has been carried out for about 30 years. Ever since it was found that systemic virus infections could be cured by meristem culture (Morel and Martin 1952) such culture has been practised for this purpose, frequently to be followed by multiplication of the virus-free shoots by micropropagation. Micropropagation, pathogen elimination and germplasm conservation *in vitro* have been practised for many years and are routine, but improvements are still being made and new methods introduced. *In vitro* tuberisation - production of very small tubers directly on *in vitro* shoots - is now sometimes used for multiplication and distribution, and as a simple method to transfer *in vitro* material to the field (Addy 1988). Embryo and anther culture are now also applied in breeding schemes for manipulation of ploidy and to aid introgression of germplasm from wild species. In addition, more sophisticated tissue culture techniques, such as protoplast culture and transformation, have enormous potential to aid breeding. The full range of *in vitro* techniques applied to potato breeding is detailed in Chapter 3.

Thus, *in vitro* manipulation includes a range of techniques of varying levels of sophistication that can be used for a great variety of purposes. However, a broad division can be

made between methods in which meristematic integrity is maintained and plants are regenerated from organised structures (meristem and nodal cultures), and those in which regeneration is via a callus phase - from single non-meristematic cells (explant cultures and protoplasts) (Fig 1.1). This division is valid in both theoretical and practical terms. From the point of view of plant development, regeneration from existing meristems is the normal mode of vegetative growth and so the *in vitro* culture of meristems involves essentially normal developmental processes. Regeneration from non-meristematic tissues, on the other hand, requires a de-differentiation of organised tissues and subsequent differentiation of meristems from the resulting callus tissue.

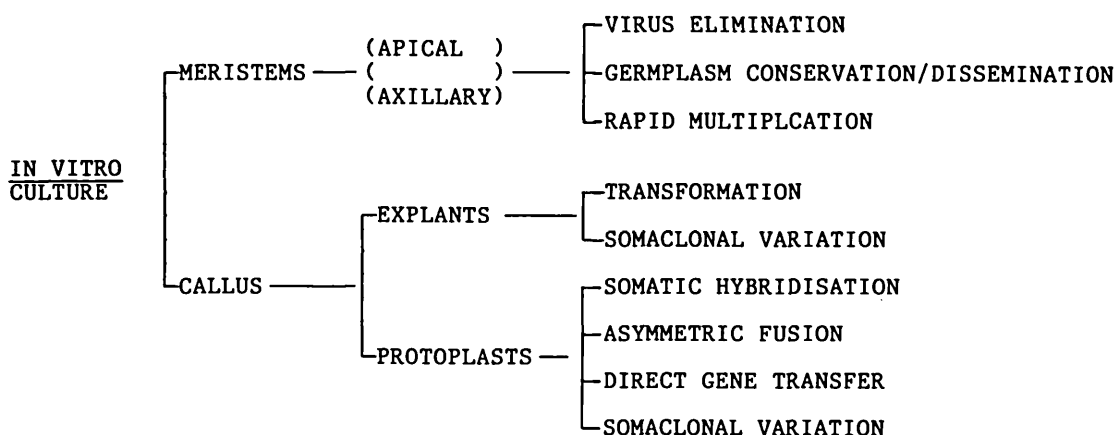


Figure 1.1 *In vitro* manipulations of potato

This shows the split between methods which retain meristematic integrity and those which regenerate from single cells. Note the different uses to which these separate methods are put.

Also, from a practical point of view, these two systems are often separated. When regeneration from callus was first established, it seemed an ideal approach for producing large numbers of plants easily and rapidly, in fact faster than by micropropagation. It was thought that all such plants would be identical, having originated from somatic tissues by mitotic cell divisions. However it was found that this was not the case, and the variation observed in such regenerant populations was termed 'somaclonal variation' by Larkin and Scowcroft (1981), whose review first drew many peoples attention to this phenomenon.

Somaclonal variation was found to be widespread in almost all populations of plants regenerated from callus, whereas populations of plants regenerated from intact meristems appeared to contain only 'true-to-type' plants. Thus a practical division between meristematic culture and tissue culture was established. If 'true-to-type' plants were required, callus should be avoided, but if new variation was required, then plant regeneration following a tissue culture cycle involving a callus phase could be employed.

1.4 Analysis of stability/variability

The apparent stability of plants regenerated from intact meristems has never been challenged and, for example, Denton et al (1977) and Wright (1983) reported stable populations after *in vitro* meristematic culture, although the regenerated plants were not tested fully. Also, with a range of *in vitro* regimes it is not always clear whether

1.4.1 Somaclonal variation

Much interest was initially focused on somaclonal variation as a new method that might be used to induce useful variation in plant breeding, in much the same way as chemical and radiation induced mutation (Shepherd et al 1980). The promise was that only one or two characters of an existing cultivar could be altered, while maintaining the favourable combination of other characters already in the plant. So analysis was made of the phenotype at whole plant or even population levels, where useful alterations in agronomic characters could be analysed directly. However, some of the changes were obviously epigenetic - probably as a result of physiological changes brought about by the tissue culture environment (eg. Wheeler et al 1985), and therefore genetic analysis would be preferable to assess the genetic stability of these plants.

Classical genetic analyses (controlled crossing studies and pedigree analysis) are quite long term. In addition they would not provide much information on the mechanisms underlying this phenomenon, and so direct studies of the genome itself were considered ideal. Cytogenetic analysis - both of chromosome structure and number - has been carried out by many people studying the causes of somaclonal variation (reviewed by Karp and Bright 1985). In general there is a relationship between the length of callus culture phase and gross changes in chromosomes, such as polyploidy, aneuploidy and chromosomal structure (eg. Karp & Maddock 1984, Fish & Karp 1987, 1988). However, even plants with apparently nor-

mal chromosomes were observed to be heritably altered in their phenotype as assessed by biochemical and molecular analysis of proteins and secondary products as well as morphological and agronomic characterisation (eg. Smith 1986, Benson & Withers 1987, Evans et al 1987). Clearly more subtle changes in the genetic make-up of the plants were taking place including changes in the DNA of the genes themselves. With the techniques of molecular genetic analysis, these changes can be identified and analysed to provide information on the mechanism and degree of variation (Brown & Lörz 1985). Examples of molecular alterations found in regenerated plants include reduction in gene copy number (Landsmann and Uhrig 1985) and changes in methylation state of the DNA (Quemada et al 1987).

1.4.2 Analysis of stability

Wherever *in vitro* culture is used to produce whole plants, functional analysis of stability takes place, simply in checking that true-to-type plants are being produced for sale or for other uses. In the production of any seed crop, removal of 'sports' and 'rogues' (any undesired plant in the field - whether a variant of the planted crop or a volunteer from another, previous crop) is common. Throughout the field multiplication of potato seed tubers, whether from micropropagated stocks or not, such undesired plants are removed. The fact that 'sports', or field variants do occur has meant that less concern has been expressed about the stability of plants produced via micropropagation. It was simply not considered to be so important, because the

plants would subsequently be checked in the field.

The functional assessment of stability described above is adequate for the production of plants for field use, although it does not really provide information on the genetic uniformity of the plants. However, when plants are maintained *in vitro* for longer periods (for example in germplasm conservation) or are distributed as *in vitro* material (*in vitro* tubers as planting material, shoots and *in vitro* tubers for distribution) then it becomes more important for the genetic integrity to be assessed. Under such circumstances, direct analysis of the genotype is much more important, since analysis of the phenotype depends on growth in constant environmental conditions (often after an acclimatisation period to remove any physiological changes due to the *in vitro* phase).

1.5 Restriction fragment analysis

Applications of the the relatively new science of molecular biology are already significantly influencing developments in modern 'high technology' agriculture. Genetic engineering of crop plants is perhaps the most visible sign of this involvement, but the most important current application is in the field of genetic analysis as an aid to conventional breeding. Probably the most powerful technique, in terms of its effect on plant breeding, is the use of restriction fragment length polymorphisms (RFLPs) as genetic markers linked to desirable traits (Tanksley et al 1989). The technique of restriction fragment analysis allows detection of

the differences in DNA sequences between individuals, which are visualised as fragments of different lengths. These differences are treated as co-dominant Mendelian markers whose positions can be mapped in crossing programmes and thus be used to select linked traits in breeding programmes. However, differences in DNA sequence are the basis of genetic variation, and so this type of analysis can also be applied to a clonal population to reveal any variant individuals. The theories and practicalities of its application to the study of genetic stability are discussed in detail in Chapter 4 of this thesis. Why it is such a powerful tool for this analysis is explained in the following section.

For analysis of genetic stability by the study of restriction fragments the first point to make is that by looking at the genome itself, problems associated with environmental interactions are eliminated. The advantage is that this allows direct comparison of plants from widely differing environments, including shoots that are still in culture. Also DNA samples are simple to store and require little space, thus enabling comparisons between starting and final material to be made directly. Second, there is considerable scope for this type of analysis of DNA. The number of markers ('probe' x enzyme combinations - see Chapter 4) is, for practical purposes, infinite. Where analysis of the phenotype is used to look at certain, small, portions of the genome (the expressed genes only), using restriction fragments the entire genome is available for analysis. However, this is also the major drawback in using this approach;

molecular analysis could only be complete by sequencing the entire genome of each plant! Thus it is not feasible to use this approach to identify positively 'identical' plants - only to assess *in vitro* methods for their effect on stability. Even so, such conclusions must be statistically based, and depend on the testing of a sufficient number of plants with enough markers to provide high levels of confidence.

Of course this does not mean that the analysis can only be used in a negative sense - only to tell if a plant is variant, not if it is normal - but, as before, levels of confidence will apply. Unless and until the mechanisms of somaclonal variation are elucidated and tests are based on the knowledge of what particular alterations are likely to be present, all study of its presence or absence will, of necessity, be based on statistical evidence.

1.6 Aims of this project

This study was set up in response to questions asked at CIP (the International Potato Centre, Lima, Peru), and by others on the genetic stability of *in vitro* stored potato. The aim was to assess stability by analysing populations of potato plants, produced by various *in vitro* regimes, for genetic variability. A specific question to be answered was whether the micropropagation and *in vitro* storage regimes currently used, produced plants identical to the parent material, or showed examples of somaclonal variation - more commonly associated with regeneration from callus cultures. Thus, as

well as plants produced by micropropagation and *in vitro* storage regimes, a population of plants regenerated from callus would be looked at in order to compare the different types of culture.

Also, since this was the first study designed to tackle this specific question, an important aspect was the development and evaluation of methods which could be used for such an analysis. Restriction fragment analysis, because of its potential for 'genetic fingerprinting', was chosen as a method to analyse the genotype, alongside methods to analyse the phenotype of the regenerated plants. A requirement was therefore the development of procedures specifically adapted to the analysis and assessment of stability, yet practically applicable to a large number of plants.

Chapter 2. Materials and Methods

2.1 Plant material

In vitro shoot cultures of cultivar Desiree were previously established in this laboratory (Foulger 1987). Other cultivars were either from lines already established in this laboratory or were provided by the Scottish Crop Research Institute (SCRI), Pentlandsfield, Scotland.

2.2 Chemicals and equipment

Chemicals used were standard AR grades, obtained from standard suppliers. Specialised chemicals and equipment are listed below.

2.2.1 Chemicals

Tissue Culture

Murashige and Skoog salts and vitamins	Flow Laboratories, Irvine, Scotland
6-Benzylaminopurine (BAP)	B-6750 Sigma Chemical Co., Poole, Dorset
Gibberellic acid (GA ₃)	G-3250 Sigma
Naphthalene-acetic acid (NAA)	N-0375 Sigma

Chlorocholine chloride (CCC)	C-2635 Sigma
Bacto-agar	Difco Laboratories Surrey
<u>Polyacrylamide gel electrophoresis</u>	
Acrylamide	A-8887 Sigma
N.N'-Methylene-bisacrylamide (Bis)	M-7256 Sigma
Tris(hydroxymethyl)aminomethane (Tris 7-9)	T-1378 Sigma
Ammonium persulphate	A-6761 Sigma
3-Dimethylaminopropionitrile (DMAP)	D-1008 Sigma
Sodium sulphite	S-0505 Sigma
Sodium bisulphite	S-9000 Sigma
Amido black (Naphthol blue-black)	N-3005 Sigma
Ethylenediamine-tetra-acetic acid (EDTA)	ED2SS Sigma
Boric acid	B-0252 Sigma

Trichloroacetic acid (TCA)	T/2950 Fisons, Loughborough, Leics
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Coomassie brilliant blue R	B-0630 Sigma
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Molecular biology

All Restriction endonucleases	Bethesda Research Laboratories, (BRL
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Sodium dodecyl sulphate (SDS)	44244 BDH Chemicals Ltd. Poole
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Ethidium Bromide	E-1510 Sigma
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Caesium chloride	C-3032 Sigma
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Deoxyribo-nucleotides (dATP etc)	Amersham Int. plc Amersham, Bucks.
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Hexa-deoxyribo-nucleotides	27-2166 Pharmacia, Uppsala Sweden
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Bovine serum albumin (nuclease-free)	510-5561 UA BRL
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Bovine serum albumin (fraction V)	A-4503 Sigma
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Bovine serum albumin (crystalline grade)	A-7638 Sigma
DNA Polymerase I large (Klenow) fragment	510-8012 SA BRL
Lambda DNA, HindIII restricted (size marker)	BRL
Øx174 DNA, HaeIII restricted (size marker)	BRL
5'-[α ³² P]dATP	Pb 204 Amersham
Yeast extract	Difco
Bacto-tryptone	Difco
Ampicillin	A-9393 Sigma
Carbenicillin	C-1389 Sigma

2.2.2 Equipment

Nalgene filter units	120-0020 Techmate Ltd, Lutor Beds.
Buraton sterilising fluid	Gibbs & Dandy,

	Luton, Beds.
Gilson 'Pipetteman' micropipettes	Anachem Ltd, Luton Beds.
Nalgene 50 ml Oak ridge polycarbonate centrifuge tubes	Techmate
Petri dishes	Sterilin Ltd, Teddington, Middx.
30 ml, 60 ml & 120 ml plastic containers	Sterilin
Miracloth	Cambridge Bioscience
Biodyne A (transfer membrane)	Gallenkamp
Protein electrophoresis equipment (Protean II cell)	Biorad Laboratories Ltd, Watford, Herts
DNA electrophoresis equipment (DNA SUB & MINISUB cell)	Biorad
Electro-blotting equipment (Trans-blot cell)	Biorad
Flow controller	FH-15A Grant Instruments, Cambridge

Cooler	FC25	Grant Instruments
Autoradiogram cassettes	400/S7	Genetic Research Instrumentation Ltd, (GRI)
Intensifying screens	409/F7	GRI
X-ray sensitive film	.	Fuji

2.3 Aseptic technique

2.3.1 Media preparation

All media used for plant *in vitro* and bacterial cultures must be sterilised before use to remove microbial contamination. The culture media used in this thesis are listed in Appendix I, with the relevant method of sterilisation. Solid media were poured (while still molten after autoclaving and subsequent addition of heat labile compounds) into sterile plastic containers (Sterilin) under aseptic conditions in a laminar flow bench. All molecular biology stock solutions were sterilised by autoclaving except where stated (Appendix I).

2.3.2 Equipment

Glassware used was sterilised overnight with dry heat at 160°C in an oven. Disposable plastic containers (Sterilin) were obtained sterile. Plastic pipette tips and microcentrifuge tubes (Eppendorf tubes) were sterilised by autoclaving. Aseptic manipulations of plant tissues were carried out in a laminar flow cabinet, previously sterilised by spraying with 'Buraton', and all tools for such work were flamed with methylated spirits.

2.3.3 Sterilising plant material

Shoot cultures were initiated from tuber sprouts and leaf disc explants were taken from pot-grown leaves. Surface sterilisation of this material was achieved by their immersion in a solution of 5-10% commercial bleach for 10-20 minutes, then washing in six changes of sterile distilled water before transfer to culture medium.

2.4 Growth and maintenance of plant material

2.4.1 *In vitro* shoot cultures

In vitro shoot cultures were initiated from tuber sprouts. After surface sterilisation, single nodes were excised and transferred to shoot propagation medium (Appendix I), in 150 ml plastic containers (Sterilin). Shoot cultures were grown in a controlled environment room, with 16 hour day 25°C and 160-180 $\mu\text{E m}^{-2} \text{ s}^{-1}$ from warm daylight fluorescent tubes.

Container lids were loosened slightly to allow diffusion of ethylene, produced by the plant material, to the atmosphere. After 4-6 weeks the shoots were subcultured by excising stem pieces containing a bud, after removing large leaves, and transferring them to fresh medium.

2.4.2 Shaker cultures

Stem pieces containing 5-6 nodes were excised from shoot cultures, large leaves were removed and they were placed into 25 ml of liquid culture medium (Appendix I) in sterile glass 250 ml Ehrlenmeyer flasks, loosely stoppered with cotton wool. Cultures were maintained on an orbital shaker at 60 rpm in a controlled environment room under the same conditions as the shoot cultures.

2.4.3 *In vitro* tuber production

4-5 week old shaker cultures were taken and the liquid medium was replaced by 25 ml of tuber induction medium (Appendix I). Cultures were then placed in the dark in the controlled environment room, with no shaking, for at least 6 weeks. *in vitro* tubers were then harvested and stored in 30 ml plastic containers (Sterilin) at 4°C.

2.4.4 Storage of shoot cultures at reduced temperatures

1-2 week old shoot cultures were taken and placed either in a cooled incubator in the dark at 4°C, or a cold room at 5-

10°C fitted with lights and a timer to give a 12 hour day. Cultures were maintained there for 6 and 12 months before removal to the controlled environment room under normal shoot culture growth conditions for regeneration and further subculture.

2.4.5 Storage of shoot cultures under osmotic pressure

Single nodes were excised from shoot cultures, large leaves were removed, and then the nodes were subcultured onto storage medium (Appendix I) in 30 ml plastic containers (Sterilin). Cultures were maintained under normal shoot culture conditions (but lids were not loosened) for six months, when portions of the tissue were subcultured onto fresh storage medium for a further six months. Separate portions of the tissue were taken at each subculture and placed into liquid medium (as described for shaker cultures) for regeneration and subsequent subculture.

2.4.6 Regeneration of shoots from explant-derived callus

Whole leaves were taken from pot-grown plants and sterilised as previously described. Discs were removed (avoiding the mid-rib) with a number six cork borer, and placed onto callus induction medium (Appendix I) in petri dishes (Sterilin) for 14 days. The discs were then transferred to shoot regeneration medium (Appendix I) in 8 cm glass jars covered with a 9 cm petri dish, sealed with Nescofilm which was ven-

tilated with 1 cm slits. Shoots were subcultured onto shoot propagation medium (Appendix I) as they regenerated from the callus pieces (4-6 weeks).

2.4.7 Transfer to compost-filled pots

To promote strong root growth, single nodes from *in vitro* shoots or shaker cultures were subcultured onto rooting medium (Appendix I) in 60 ml plastic containers (Sterilin) for 4 weeks under normal shoot culture conditions. The medium was then gently washed from the roots and the plantlet was placed in soaked EFF compost in 9 cm plastic plantpots without saucers. Pots were placed in propagators, to maintain high humidity, in controlled environment cabinets ($450 \text{ uE m}^{-2} \text{ s}^{-1}$ from warm fluorescent and tungsten lights, 12 hour day, 20/16°C, 70/80% relative humidity) until good root growth was established when they could be taken out of the propagators. After 4 weeks the plants were potted on into 15 cm pots and transferred to a greenhouse with a 10 hour day (20/16°C) to promote tuberisation. Tubers were harvested after the plants had senesced naturally and were stored in linen bags at 4°C.

Pot plants were also initiated from *in vitro* tubers which had been allowed to sprout at 25°C before being placed in 9 cm pots in the same way as the *in vitro* shoots. Larger (pot-grown) tubers were allowed to sprout in linen or paper bags before being potted directly into 15 cm pots and placed in the greenhouse.

2.5 Polyacrylamide Gel Electrophoresis of Proteins

Soluble tuber proteins were analysed on non-denaturing polyacrylamide gels according to the method of Stegemann and Schnick (1982).

2.5.1 Sample preparation

Tubers, freshly harvested or stored in linen bags in a cold room at 4°C, were washed and weighed before homogenising in a Waring blender. The homogenate was squeezed through two layers of muslin and 1.2 ml of the sap was added to 10 µl of extraction buffer (Appendix I) in an Eppendorf tube. Starch was pelleted in a microcentrifuge and 0.9 ml of the supernatant was transferred to a fresh Eppendorf tube containing 0.1 ml of sucrose loading buffer (Appendix I), vortexed briefly and then stored at -20°C until needed.

2.5.2 Gel preparation

Gels were prepared and run in a Biorad Protean II Slab Gel Electrophoresis Unit coupled to a cooling system (Grant Instruments) to maintain a running temperature of 4°C. The casting apparatus was assembled to enable four gels to be run at one time. 200 ml of acrylamide solution (Appendix I) was degassed before addition of the other components (Appendix I) and carefully poured between the plates. Gel combs were inserted and the gel was allowed to polymerise at room temperature.

2.5.3 Gel operation and staining

The gel apparatus was assembled and running buffer (Appendix I) poured into the top and bottom reservoirs. 30 μ l of thawed sample were loaded into each well and the gels run at 30 mA per gel (4 gels = 120 mA) until the marker dye was 3-4 cm from the bottom of the gel. The gels were stained for at least 4 h in Coomassie blue and destained for 24 h in 3-4 changes of destain solution (Appendix I). Stained gels were photographed.

2.6 Agarose Gel Electrophoresis of DNA

Submerged agarose gels were used routinely to assess the quality and quantity of isolated and restricted DNA and also to produce filters for DNA hybridisation. DNA Sub-, MiniSub-, and Wide MiniSub-cells (Biorad) were used with matching UV transparent gel trays and combs.

Powdered Agarose (type II medium EEO) was weighed and boiled, in a microwave oven, in the appropriate amount of electrophoresis buffer until completely dispersed. Gel trays were sealed with tape and combs positioned with the teeth 0.5 - 1 mm from the bottom of the tray. The molten agarose was poured into the tray after it had cooled to around 55°C and allowed to set at room temperature. Once set, the tape and comb were removed and the tray (with gel) placed into the tank and covered with a known volume of buffer. DNA samples (containing glycerol loading buffer) were loaded into the wells and the gel run at constant current for an appropriate time, depending on the size of the

gel and the particular circumstances.

Gels were stained in 0.5 mg ml⁻¹ ethidium bromide for 30 to 60 minutes, depending on the size of the gel, and DNA was visualised on a UV transilluminator. Permanent records were produced by photographing the gel on the transilluminator with a Polaroid MP4 system and type 660 film, using a red filter to block out the UV.

2.7 DNA Isolation

2.7.1 Plant genomic DNA: Method 1

This method is based on personal communication from Dr. B D Hall (PBI Cambridge).

4-8 g of leaves or tuber sprouts were taken, frozen in liquid nitrogen and ground in a coffee grinder (Moulinex) for 2x30 seconds in more liquid nitrogen. Tissue was transferred (while still frozen) to a 50 ml centrifuge tube containing 15 ml of extraction buffer I (Appendix I), mixed well and incubated at 42°C for 45 minutes. Samples were cooled to 20°C before extracting with 20 ml of phenol:chloroform (1:1). Layers were resolved by centrifuging at 3000 rpm and the aqueous phase removed to a fresh tube containing 20 ml of chloroform:iso-amyl alcohol (1:1). After mixing well the layers were again resolved by centrifugation at 3000 rpm for 5 minutes and the aqueous layer removed to a clean tube. An equal volume of isopropanol was added and the DNA allowed to precipitate over-

night at 4°C. The tube was then centrifuged at 3000 rpm for 10 minutes and the pellet drained well before resuspending in 10 ml of half-strength TE. Once resuspended, this was made up to full strength TE (with stock solutions), then 0.1ml RNase solution (Appendix I) was added and incubated at 37°C for one hour. Phenol:chloroform and chloroform:iso-amyl alcohol extractions were performed as before, and 1/20 volume of Sodium acetate was added before precipitation with an equal volume of iso-propanol overnight at 4°C as before. The DNA was pelleted by centrifugation at 3000 rpm for 10 minutes, drained well and resuspended in 4 ml TE.

2.7.2 Plant genomic DNA: Method 2

This method has been adapted from the method of Dellaporta et al (1983).

1-2 g of leaves or *in vitro* shoots were ground in liquid nitrogen in a mortar and pestle to a fine powder. The powder was transferred, while still frozen, to a 50 ml tube containing 15 ml of extraction buffer II (Appendix I). 1 ml of 20% SDS was added to the tube, which was incubated at 65°C with occasional shaking for 10 minutes. 5ml of 5M potassium acetate were added, with shaking, and the tube placed in ice for 20 minutes. After centrifugation at 10,000 rpm for 20 minutes the contents were poured through Miracloth into a clean tube containing 10 ml of isopropanol. This was incubated at -20°C for one hour before centrifugation at 12,000 rpm for 20 minutes. The supernatant was poured off and the tube drained for a few minutes before

resuspending the pellet in 0.7 ml of 50 TE (Appendix I). This was transferred to an Eppendorf tube containing 7 μ l of RNase (Appendix I) and incubated at 37°C for one hour. 75 μ l of 3M Sodium acetate were added and the sample centrifuged in a micro-centrifuge at high speed for 15 minutes. The supernatant was transferred to a clean Eppendorf tube and the DNA precipitated with 0.5 ml isopropanol at room temperature. After 5 minutes, the DNA was pelleted in a microcentrifuge at high speed for 10 minutes and the supernatant poured off. The pellet was washed with 75% ethanol before being dried in a vacuum desiccator, followed by resuspension in 200 μ l of TE overnight at 4°C.

2.7.3 Plasmid DNA: 'Midi' scale preparation

40 ml of L-broth (Appendix I) (with appropriate antibiotics) in a conical flask were inoculated with bacteria from a single colony (of E. coli) and incubated overnight at 37°C with shaking. Bacteria were pelleted at 6000 rpm for 5 minutes at 4°C and then resuspended in 3.4 ml glucose-TE (Appendix I). 2 ml of lysozyme solution I (Appendix I) was added, mixed gently and kept on ice for five minutes before addition of 10.8 ml of alkaline-SDS (Appendix I), mixing and a further 5 minutes on ice. The solution was neutralised by the addition of 2 ml of neutralising solution (Appendix I) and then centrifugation at 15,000 rpm 4°C for 15 minutes. The supernatant was collected and added to 11 ml isopropanol, kept at room temperature for 15 minutes before centrifuging at 10,000 rpm 20°C for 30 minutes. The supernatant was discarded and the pellet resuspended in 3 ml TE.

Insoluble material was pelleted at 5000 rpm for 5 minutes and the supernatant was added to 3 g CsCl_2 in a polyallomer ultra-centrifuge tube. Once the CsCl_2 had dissolved, 75 μl EtBr were added and the tube capped, filled with 50% CsCl_2 solution (Appendix I) and spun in an ultra-centrifuge (TFT 80.4 rotor) at 66,000 rpm for 5 hours or 55,000 rpm for 14 hours. The DNA was visualised under UV and the lower (plasmid) band harvested using a hypodermic needle inserted through the side of the tube. The EtBr was removed by extraction with water-saturated butanol and the CsCl_2 was removed by dialysis. The DNA solution was made up to 400 μl and precipitated with 40 μl of 4M NH_4OH and 1 ml of ethanol at -20°C overnight. After a 15 minute spin in a microcentrifuge, the DNA pellet was dried in a vacuum desiccator and resuspended in 50 μl of SDW.

2.7.4 Plasmid DNA: Mini- scale preparation

This method was derived from Birnboim and Doly (1979).

Selected colonies (of *E. coli*) were grown as streaks on an L-plate (Appendix I) with appropriate antibiotics overnight at 37°C . Bacteria were scraped off into an Eppendorf tube and resuspended in 100 μl of lysozyme solution II (Appendix I), then incubated on ice for 30 minutes. 200 μl of alkaline SDS (Appendix I) were added, the tube vortexed gently and incubated on ice for 5 minutes. 150 μl of 3M sodium acetate were added and mixed gently by inverting, then kept on ice for 60 minutes. The tube was spun for 5 minutes in a microcentrifuge and 400 μl of the (clear) su-

pernatant was transferred to a clean Eppendorf tube. 1 ml of cold ethanol was added and the tube kept at -20°C for 30 minutes before pelleting the DNA in a microcentrifuge for 2 minutes. The supernatant was removed with a pipette and the pellet resuspended in 100 µl of sodium acetate/Tris (Appendix I). The DNA was re-pelleted with 2 volumes of cold ethanol at -20°C for 10 minutes, and resuspended in 50 µl of SDW.

2.8 Restriction Fragment Analysis

This procedure involves many individual methods and techniques taken from many different sources, but the basic methods can be found in Maniatis et al (1982).

2.8.1 Restriction of total plant DNA

30 µl of each extracted (total) plant DNA sample was cut with 20 units of restriction endonuclease (BRL) in a final volume of 100 µl using the recommended buffer with 2 µl of 0.1M spermidine tri-hydrochloride. Digestion was allowed to proceed overnight at 37°C and complete digestion was checked by removing 5 µl of the reaction mixture and running on a 0.8% agarose gel in TAE buffer (Appendix I). The reaction was stopped by the addition of 2 µl of 0.5M EDTA and the restricted DNA pelleted by the addition of 50 µl of 3M sodium acetate and 300 µl of isopropanol. After 5 minutes at room temperature the DNA was pelleted in a microcentrifuge for 5 minutes, the pellet washed with 75% ethanol, dried briefly in a vacuum desiccator and

resuspended in TE. The final resuspension volume was determined by the amount of DNA in each sample as determined from the gel of the restriction reactions. It was adjusted in order to achieve similar final DNA concentrations so that equal amounts of DNA could be loaded on subsequent gels. Once thoroughly resuspended, glycerol loading buffer (Appendix I) was added (1/3 volume) and the amount of DNA checked by running 2 μ l of each sample on another 0.8% agarose gel in TAE. Samples were stored at -20°C.

2.8.2 Production of filters - Blotting

Restricted plant DNA samples (volumes adjusted to give equal quantities of DNA) were loaded into a 25 cm gel (200 ml 0.75% agarose) in 1500-1600 ml of TBE buffer (Appendix I) in a DNA Sub-Cell (Biorad). Gels were run - routinely - at 50 mA for 13 hours but currents and times were sometimes altered to allow for particular circumstances. After running, the gels were stained in 0.5 μ g ml⁻¹ EtBr for 45-60 minutes before visualising the DNA on a UV transilluminator. Gels were trimmed to 20 cm long, usually removing the top 5 cm, photographed and exposed to the UV light for 30 minutes. The DNA was then denatured by soaking the gel in denaturing solution (Appendix I) for one hour, with shaking, and neutralised in 3M sodium acetate for another hour. This was followed by two 20 minute washes in electro-transfer buffer (Appendix I). Electro-transfer (or electro-blotting) onto Biodyne A membranes was performed in a Biorad Trans-Blot apparatus, according to the protocols described in the Biodyne handbook. A sandwich of fibre pad ; Whatmann 3MM

paper ; membrane ; gel ; Whatmann 3MM paper ; fibre pad was constructed (with the Whatmann paper and the membrane cut to a size just larger than the gel). Air bubbles were squeezed out with a pipette, and the sandwich was placed into the transfer tank in the gel holder supplied, ensuring that the gel was on the negative side and the membrane on the positive side of the tank. The tank was filled with transfer buffer (Appendix I) and transfer carried out at 10 V (200-300 mA) for 3-4 hours followed by 20 V (600-1200 mA) for 1-2 hours. The apparatus was cooled by a cooling system (Grant Instruments) through a coil in the tank. After transfer the membrane was dried in air for 30 minutes before baking at 80°C for one hour.

2.8.3 Probe preparation and labelling

Plasmid DNA, containing potato DNA as probe, was taken and the insert was excised from the plasmid sequences using the appropriate restriction endonuclease. Insert and plasmid were separated by electrophoresis through 1-2% low-gelling-temperature agarose gels in TAE containing 0.5 $\mu\text{g ml}^{-1}$ EtBr. Pieces of gel containing the required insert DNA were excised from the gel (visualised on UV transilluminator), weighed and boiled for 7 minutes in 3 ml of SDW per gramme of gel. The solution was then stored at -20°C.

Probe DNA was labelled by the method of Feinberg and Vogelstein (1983) often referred to as 'oligo-labelling'. Approximately 100 ng of DNA to be labelled were taken, made to 80 μl with SDW and boiled for 3 minutes before being plunged into ice. 20 μl of OLB (Appendix I) and 4 μl of BSA

(BRL) were added and the mixture transferred to a siliconised Eppendorf tube in which 100 uCi of ^{32}P dATP (Amersham) had been dried down. The contents of the tube were mixed gently (still on ice) before addition of 4-6 units of Klenow fragment (DNA polymerase 1 large fragment) and incubation overnight at room temperature. Labelled DNA was separated from unincorporated nucleotides by passage through a column containing Ultrogel 34. Fractions containing labelled DNA were pooled and used immediately for hybridisation.

2.8.4 Hybridisation: Method 1

Hybridisation was carried out as suggested in the Biotek handbook, method A, but using a Hybaid I system of bags and nylon meshes. Up to 4 filters - each separated by a nylon mesh - were sealed inside a PVC bag and pre-hybridisation solution (Appendix I) was added through one of the ports. Air was slowly removed from the bag until the solution completely covered the filters and then the bag and filters were incubated in a shaking water bath at 65°C for at least one hour. The bag was then inflated and the prehybridisation solution was poured off, mixed with the boiled probe and returned to the bag. The bag was inverted 5-10 times to ensure even mixing of the radioactive probe and the air was withdrawn as before, carefully to ensure no air bubbles remained in contact with the filters. The filters were hybridised at 65°C in a shaking water bath for at least 16 hours before removing the hybridisation solution as described previously. The filters were washed by drawing

salt solutions through the bag across the surface of the filters, starting with 250 ml of 2xSSC 0.2%SDS then 250 ml of 1xSSC 0.2%SDS and finally 250 ml of 0.5xSSC 0.2%SDS (Appendix I). After this the bag was cut open and the filters removed and placed in a sandwich box with 500 ml of 0.2xSSC 0.2%SDS and washed at 60°C for 30 minutes. The filters were then wrapped in cling film and exposed to Fuji X-ray sensitive film in autoradiogram cassettes with intensifying screens (GRI Ltd) for 1 day to 2 weeks. Films were developed using Kodak developing and fixing solutions.

2.8.5 Hybridisation: Method 2

This is taken from Church and Gilbert (1983) and is carried out in Hybaid bags as before, but without the nylon meshes. 2 filters (washed in SDW before use) were placed back-to-back (DNA side facing outwards) in the bag and sealed. 20 ml of Church buffer (Appendix I) were added, air removed from the bag and the bag incubated at 65 °C for 10 minutes. The solution was then removed and replaced with 10 ml of Church buffer containing denatured probe. The solution was spread evenly through the bag with a glass plate and the air removed as before. Hybridisation was carried out at 65 °C for at least 24 hours, with shaking. After this the probe solution was replaced by 25 ml of Church wash solution and agitated for 5 minutes before the solution was poured off. This was repeated 2 more times before the bag was cut open and the filters washed in a sandwich box at 60 °C in the same wash buffer. Autoradiography was performed as in the previous method.

2.8.6 Hybridisation in sandwich boxes

To hybridise many filters (up to 24 at one time) 100 ml of pre-hybridisation solution (Church) was placed in a sandwich box and the filters placed into this one by one, ensuring that the surface of the filter was covered before adding the next filter. The box was incubated at 65°C (in a water bath) for 10 minutes and then the filters were removed (one at a time) and placed in a second sandwich box containing 100 ml of hybridisation solution (containing probe). Filters were placed in the box with the DNA side uppermost and - again - complete coverage of the filter with the solution was ensured. The box was sealed before incubation overnight at 65°C with shaking when the filters were removed from the box, and then replaced one at a time as before to re-cover the filters with the hybridisation solution. After a second overnight incubation at 65°C the filters were washed by placing into other sandwich boxes containing 500 ml of wash solutions, and incubation at appropriate temperatures. Autoradiography was performed as previously stated.

2.8.7 Stripping of filters - for re-use

In order to re-hybridise filters with many different probes, bound probe must be removed by washing in very low salt buffers at high temperatures. After autoradiography, filters were stored frozen at -20°C, still wrapped in cling film so that they did not dry completely and the probe bound

irreversibly. To strip, the filters were thawed, unwrapped and placed into large (at least 50 ml per filter) volumes of strip solution (Appendix I) in sandwich boxes. These were incubated at 80°C for one hour, with shaking, and then the solution was replaced with fresh strip solution and the filters washed for a further hour. After a further hours wash in distilled water the filters were ready for pre-hybridisation and subsequent hybridisation again. If not required immediately for this, then the filters were again stored at -20°C.

Chapter 3. *In vitro* manipulation of potato

3.1 Introduction

The potato, which is typical of the *Solanaceae* as a group, is amenable to most types of *in vitro* manipulation. For example, single meristems (apical or axillary) can be excised and cultured, with or without stem pieces, as a routine procedure. Tissue culture of explants from almost any plant organ is also straightforward, and in many cases, plants can be regenerated from protoplasts. This, coupled with the difficulties of conventional breeding of a highly heterozygous, tetraploid crop which is vegetatively propagated, have resulted in the potato becoming the crop plant to which *in vitro* culture techniques have been applied most extensively.

These aspects have been reviewed in detail by Wang and Hu (1985) and Dodds (1988). The most widely applied techniques are described briefly here. In the results section of this chapter details are given of the *in vitro* culture, and the plants produced for further analysis are listed.

3.1.1 Meristem culture and micropropagation

Intact meristems of potato can be cultured for a variety of uses. For virus elimination, very small explants (>1mm) with little or no extra tissue apart from the meristem, are cultured in order to regenerate plants free of systemic viruses (Wright 1983). Serial subculture of larger stem



Figure 3.1 Stem explant with single axillary meristem

This is the standard form of serial subculture used for micropropagation, with the regeneration of the shoot from an intact axillary meristem. Note the basal callus formed at the cut end of the stem. Care must be taken to ensure that if shoots regenerate from such callus they are not subcultured further.

explants, that include single apical or axillary meristems, (Figure 3.1) is a means of rapid multiplication whilst maintaining the virus-free status of the material (Hussey and Stacey 1982). This is now a routine method of micropropagation for potato, used by many seed tuber producers (Jones 1988). Propagation can be carried out in liquid or on solid media, and by using whole stems (4 - 5 nodes) in shaken liquid cultures very high multiplication rates can be achieved (up to 2.5^{17} per year claimed by Wang and Hu, 1985).

The media used are usually based on that of Murashige and Skoog (1963) with 2 - 3% sucrose, often with low levels of some phytohormones (cytokinins or gibberellins, auxins are avoided because they promote callus proliferation). Components of the media can be altered (as can temperature of

culture) in order to reduce the growth rate of plantlets and so increase the time interval between subcultures. This provides a simple method for long-term preservation of cultures - required for germplasm conservation. Complete cessation of growth by ultra-low temperatures (cryopreservation) is also possible, but is not yet fully developed (Withers 1987).

Similarly, by altering media components (high levels of sucrose and cytokinins), small *in vitro* tubers can be produced (Hussey and Stacey 1984, Wang and Hu 1982). These 'micro' tubers offer a simple, practical method for transport of sterile material and a possible method for large scale field planting, direct from culture (Wiersema et al 1987). At present the establishment in soil of *in vitro* plantlets requires careful attention to detail. Plants must be transferred to high humidity conditions for the first week after transplanting into soil, with care to avoid pathogens and water stress.

3.1.2 Tissue culture involving a callus phase

Whole plants can be regenerated from non-meristematic tissues of a wide range of potato genotypes. However there are strong genotype/media interactions which implies that there are genes controlling the tissue culture response (Fish and Jones 1988). The induction and maintenance of callus, and the regeneration of shoots by organogenesis from callus can be controlled by levels of phytohormones in the growth media. A two-step procedure for the production,

first of callus from explants, and then shoots from the callus is typical (eg. Wheeler et al 1985). Auxins and cytokinins are usually required for the induction of callus, whereas cytokinins and gibberellic acid are used most often for organogenesis. Callus cultures can be readily maintained on media containing auxins, and suspension cultures of rapidly dividing cell clusters in liquid medium are convenient sources of potato tissue.

3.1.3 Culture and use of protoplasts

Protoplasts can be isolated from callus and suspension cultures, but most commonly for potato, they are usually isolated from organised plant tissues, because the callus and suspension cultures are prone to somaclonal variation (Jones 1987). The cell wall is degraded with enzyme mixtures (cellulases and pectinases) in slightly hypertonic solutions to prevent bursting by osmotic shock. After washing to remove the wall-degrading enzymes, the protoplasts are cultured in supplemented media with hormones, and induced to re-form cell walls (Foulger and Jones 1986). As they divide, the osmoticum is reduced and small colonies form, from which shoots are induced in a similar way to other callus cultures.

Freshly isolated protoplasts can be used for many manipulations. By applying chemical treatments or electric pulses, partial breakdown of the membranes can be induced. This enables transformation by uptake of DNA in the surrounding medium (direct gene transfer), which can be used

for studies on transient expression of DNA constructs as well as for stable integration of the inserted DNA (e.g. Jones et al 1987, Uchimiya et al 1986). Fusion of touching protoplasts also occurs after such breakdown of membranes (Templaar and Jones 1985). Such protoplast fusion can be achieved between mixtures of protoplasts from different tissues, plants, plant varieties or even species (e.g. Fish et al 1988, Fahleson et al 1988, Jones 1988b). Fusion can be applied to combine the whole genomes of two viable populations to produce true somatic hybrids, or when one population is treated (often by irradiation) to induce partial genome transfer or transfer of only cytoplasmic genes (asymmetric hybridisation or cybridisation, respectively) (e.g. Dudits et al 1988, Vardi et al 1987, Jones 1988b).

3.1.3 Anther and embryo culture

Culture of haploid microspores within anthers and the subsequent regeneration of plants from microspore-derived callus, is a useful method for reducing ploidy levels. This is an important step in modern potato breeding. Breeding at the dihaploid ($2n = 24$) level is much simpler than at the tetraploid level, and the subsequent resynthesis of tetraploids by protoplast fusion or via unreduced gametes involves addition rather than segregation of desired traits. It is also possible to produce monohaploids ($n = 12$) and true homozygous diploids by anther culture. These can be crossed with other diploids, and then fused to produce tetraploids with maximum heterozygosity - another goal of potato breeding. The theory and practical use of anther

culture and breeding at reduced ploidy levels is reviewed in Ross (1985) and others (Wenzel and Uhrig 1981, Fish 1988, Diemling et al 1988).

Another well established technique in potato breeding is embryo culture. There are many wild *Solanum* species, and the simplest approach to introduce useful germplasm is by making a sexual cross. However, in many wide species crosses, normal seed development aborts due to incorrect endosperm complement (Johnson et al 1980). Some of these aborted crosses can be rescued by excision and culture *in vitro*, or by fertilisation *in vitro* of cultured ovules (eg. Chavez et al 1988).

3.2 Results - production of plants from *in vitro* regimes

Four different *in vitro* treatments were used to provide plants for analysis, with a number of plants produced from each treatment. Experimental details of the treatments can be found in Chapter 2, and media recipes are provided in Appendix I. The general scheme followed for the production of plants is shown in Figure 3.2. The plants produced are documented in Tables 3.1, 3.2 and 3.3. The generation of the source material that was used for the further analysis of these plants is described and is also given in these tables, for each method of analysis.

3.2.1 Micropropagation

Six shoots of c.v. Desiree (obtained from one culture vessel and so derived from one original shoot) were subcultured as individual clones through six cycles over an eight month period. A multiplication rate of 6x would have been easily possible at each subculture, which could - potentially - have produced over a quarter of a million

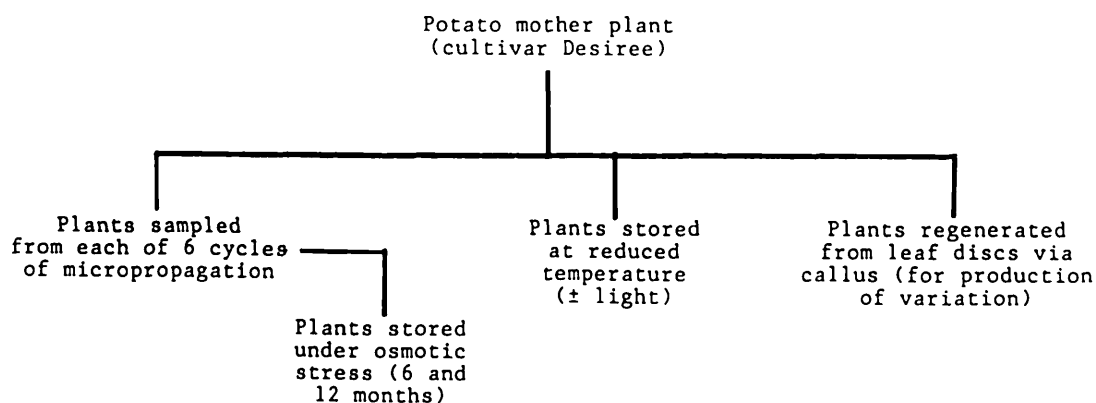


Figure 3.2 Scheme for production of plants for analysis

This shows the four groups of plants used for this study, and their derivation from the original shoot. See text for details of the treatments and the number of plants in each group.

plants! This number of plants is far too many to handle (even simply to produce without analysis), so the majority were discarded and the number of shoots was roughly doubled at each subculture to provide 20 - 25 shoots per line at the end of the period. By numbering the shoots sequentially at each subculture (A1, A2, etc. A1.1, A1.2, etc.) the provenance of each shoot could be followed.¹

3.2.2 Cold storage

After the second round of subculture, 4 shoots per line were transferred into cold storage, 2 per line in dark conditions at 4°C, and the other two in light conditions with 12 hour days at 5 - 10°C. After 6 months one shoot per line from both dark and light treatments (x.D6 and x.L6 respectively) were regenerated. After a further 6 months (12 months in all) the dark stored shoots were dead, but the remaining shoots from the light treatment were regenerated (x.L12). These regenerated shoots were multiplied (one subculture) then shaker cultures were set up to provide tissue for further analysis.

3.2.3 Osmotic storage

A single shoot from each line was taken after the 6th round of subculture and 4 nodes were subcultured onto media for storage under osmotic stress (Sx1 - Sx4). After 6 months the shoots appeared quite abnormal, with scale-like leaves and very short internodes (Figure 3.3). Portions were subcultured into shaker cultures for regeneration (Figure 3.4).

3.2.4 Callus-derived regenerants

Leaf-discs were taken from the 6 initial (pot grown) shoots and were cultured on a callusing medium and then on a shoot regeneration medium as described in Chapter 2. Many shoots were produced by this system, but only 10 shoots per line

were subcultured for further analysis.



Figure 3.3 (above left) Osmotically stored shoot

Shoot after six months on osmotic storage media. Note the scale-like leaves and the almost complete absence of internode elongation.

Figure 3.4 (above right) Shaker culture

Portion of osmotically stored shoot three weeks after transfer to liquid media. Note the large number of axillary meristems now available for further subculture. Although some callusing of the original tissue was observed, shoot regeneration was from existing meristems.

3.3 Treatment of plants from *in vitro* regimes

The shoots produced by micropropagation and regenerated from leaf-disc callus, were rooted, potted into compost filled pots and grown in controlled environment cabinets and green-houses to provide material for the analysis of stability. Leaf tissue was taken and washed before use as source

material for DNA analysis. Tissue not used immediately was frozen in liquid nitrogen and stored at -70°C . Tubers were harvested from these pots, and stored in linen bags at 4°C . Some of these tubers were used for protein analysis and others for planting material for the field trial. Others were used to provide a second pot-grown generation, tubers from which were used for protein analysis and as planting material for the field trial.



Figure 3.5 (above left) In vitro tubers produced in flask
In vitro tubers produced after six weeks in flask with tuber induction medium.

Figure 3.6 (above right) Harvested in vitro tubers
These were stored at room temperature as shown until required.

Source material for DNA analysis of the stored shoots was taken from the shaker cultures produced from these shoots. This material was treated as before, i.e. washed before use, frozen in liquid nitrogen and stored at -70°C if not used immediately. The shaker cultures were grown further and used to produce *in vitro* tubers (Figures 3.5 & 3.6). Some of these *in vitro* tubers (those from the osmotically stored shoots) were planted into compost filled pots. Tubers from these pot-grown plants were used for protein analysis and as planting material for the field trial. The *in vitro* tubers from the cold stored shoots could not all be grown in pots, due to a long dormancy. These shoots were thus not analysed by tuber protein analysis, and *in vitro* tubers from these shoots were planted directly into the field.

3.4 Discussion

This chapter contains the results of the *in vitro* manipulations, and in it details are given of the production of the source material used for the stability analyses described in following chapters. As can be seen from the tables at the end of this chapter, not all the plants were analysed by all the methods, and so the numbers of plants from each treatment looked at is different for each type of analysis. However, overall there were about 130 plants in the micropropagated group, 50 plants regenerated from leaf disc callus and 20 plants from each of the two storage regimes analysed.

It appears that light is required for long term storage at

reduced temperatures, as the shoots stored in the dark at 4°C did not regenerate after 12 months under these conditions. Storage under osmotic stress produces a highly abnormal appearance, but does not seem to affect regeneration adversely. At the International Potato Centre (CIP) a combination of cold temperature (8°C) and osmotic stress is used for long term storage of potato shoot cultures. Under this regime, the period between subcultures has been extended to .3 years for some genotypes (J Dodds, personal communication).

The use of *in vitro* tubers for storage and transfer to soil is attractive and becoming more widespread (Jones 1988a). The long dormancy of some of these tubers is a problem in this respect, but this is perhaps due to the storage conditions of these tubers (room temperature). Cold temperature 'vernalisation' or other treatments may be useful in breaking this dormancy.

1. Because the required number of shoots was obtained before the final subculture for some lines, these were not multiplied, but subcultured on a one-for-one basis. These were not given a further number, and so have only a five-figure code as opposed to a six-figure code for the other shoots.

Key to tables:

Plant name

Unique code number given to each plant

Type

MP - micropropagated plant

CST - cold stored plant

OST - plant stored under osmotic stress

REG - plant regenerated from leaf-disc callus

Tissue for DNA analysis

p/g - leaves from pot-grown plants

i/v - whole shoots from shaker cultures

Filter name

Code for filter used for restriction fragment analysis.

Restriction enzyme used is also specified for individual filters (replaces () in table 3.3)

Refer to Appendix II for results grouped by filter name

Tubers for protein analysis and field trial

p/g 1 - tubers from first pot-grown generation

p/g 2 - tubers from second pot-grown generation

i/v - *in vitro* produced tubers

Table 3.1
List of plants regenerated
after micropropagation

Plant name	Type	Tissue for DNA analysis	Filter name	Tubers for protein analysis	Tubers for field trial
A2111.1	MP	p/g	AB I	p/g 2	p/g 2
A2111.2	MP	p/g	AB II	p/g 2	p/g 2
A2113.1	MP			p/g 1	p/g
A2113.2	MP	p/g	AB II	p/g 1	p/g 2
A2113.3	MP	p/g	AB I		
A2123.1	MP	p/g	AB I	p/g 1	p/g 2
A2511.1	MP	p/g	AB II	p/g 2	p/g 2
A2511.2	MP	p/g	AB I	p/g 1	p/g 2
A2512.1	MP	p/g	AB I	p/g 1	p/g 2
A2512.2	MP	p/g	AB II	p/g 2	p/g 2
A2521.1	MP	p/g	AB I	p/g 2	p/g 2
A63111.1	MP	p/g	AB I	p/g 2	p/g 2
A63111.2	MP	p/g	AB I	p/g 1	p/g 1
A63112.1	MP	p/g	AB II	p/g 2	p/g 2
A63112.3	MP	p/g	AB II	p/g 2	p/g 2
A63312.1	MP	p/g	AB II	p/g 2	p/g 2
A63321.1	MP	p/g	AB I	p/g 1	p/g 1
A63321.2	MP	p/g	AB II	p/g 2	p/g 2
A63322.2	MP	p/g	AB I	p/g 1	p/g 1
A63322.3	MP	p/g	AB I	p/g 1	p/g 1
B1211.1	MP	p/g	AB I	p/g 1	p/g 1
B1211.2	MP	p/g	AB I	p/g 2	p/g 2
B1213.1	MP			p/g 1	p/g
B1221.1	MP			p/g 1	
B1221.2	MP	p/g	AB II	p/g 2	p/g 2
B1222.1	MP	p/g	AB I	p/g 2	p/g 2
B1222.2	MP	p/g	AB I	p/g 1	p/g 1
B1522.2	MP	p/g	AB II	p/g 2	p/g 2
B15231.1	MP	p/g	AB I	p/g 1	p/g 1
B15231.2	MP	p/g	AB II	p/g 2	p/g 2
B1531.1	MP	p/g	AB I	p/g 1	p/g 2
B1531.2	MP	p/g	AB II	p/g 2	p/g 2
B1533.1	MP	p/g	AB I	p/g 1	p/g 1
B1533.2	MP	p/g	AB I	p/g 2	p/g 2
B4222.1	MP	p/g	AB I	p/g 1	p/g 2
B4222.2	MP	p/g	AB I	p/g 1	p/g 1
B4231.1	MP	p/g	AB II	p/g 2	p/g 2
B4231.2	MP	p/g	AB I	p/g 1	p/g 1
B42321.1	MP	p/g	AB I		p/g 2
B42321.2	MP	p/g	AB I	p/g 2	p/g 2
B4413.2	MP	p/g	AB II	p/g 2	p/g 2
B4431.1	MP	p/g	AB II	p/g 2	p/g 2
B4432.2	MP	p/g	AB I	p/g 1	p/g 1
C3312.1	MP	p/g	CD II	p/g 1	p/g 2
C3312.2	MP			p/g 1	p/g
C3313.1	MP	p/g	CD II	p/g 2	p/g 2
C3313.2	MP	p/g	CD I	p/g 1	p/g 1
C3332.1	MP	p/g	CD I	p/g 1	p/g 1
C3332.2	MP	p/g	CD I	p/g 1	p/g 1

C3333.1	MP	p/g	CD I	p/g 1	p/g 1
C3333.2	MP	p/g	CD I	p/g 1	p/g 1
C3511.2	MP	p/g	CD II	p/g 2	p/g 2
C3513.2	MP	p/g	CD II	p/g 2	p/g 2
C3521.1	MP	p/g	CD II	p/g 1	p/g 2
C3521.2	MP	p/g	CD II	p/g 2	p/g 2
C3522.1	MP	p/g	CD II	p/g 1	p/g 2
C6411.2	MP	p/g	CD II	p/g 1	p/g 2
C6413.2	MP	p/g	CD I	p/g 1	p/g 2
C6432.1	MP	p/g	CD I	p/g 1	p/g 2
C6432.2	MP	p/g	CD II	p/g 2	p/g 2
C6512.1	MP	p/g	CD II	p/g 2	p/g 2
C6512.2	MP	p/g	CD I	p/g 1	p/g 1
C6513.1	MP	p/g	CD II	p/g 2	p/g 2
C6513.2	MP	p/g	CD II	p/g 2	p/g 2
C6521.1	MP	p/g	CD II	p/g 2	p/g 2
C6521.2	MP	p/g	CD II	p/g 1	p/g 2
D3111.2	MP	p/g	CD I	p/g 2	p/g 2
D3113.2	MP	p/g	CD I	p/g 1	p/g 1
D3121.2	MP	p/g	CD II	p/g 2	p/g 2
D3123.1	MP	p/g	CD II	p/g 2	p/g 2
D3123.2	MP	p/g	CD II	p/g 2	p/g 2
D3512.1	MP	p/g	CD I	p/g 1	p/g 1
D3512.2	MP	p/g	CD II	p/g 2	p/g 2
D3521.1	MP	p/g	CD II	p/g 2	p/g 2
D3523.1	MP	p/g	CD II	p/g 2	p/g 2
D3523.2	MP	p/g	CD I	p/g 1	p/g 1
D5221.2	MP	p/g	CD II	p/g 1	p/g 2
D5223.1	MP	p/g	CD I	p/g 2	p/g 2
D52241.1	MP	p/g	CD I	p/g 1	p/g 1
D52241.2	MP	p/g	CD II	p/g 2	p/g 2
D52242.1	MP	p/g	CD I	p/g 1	p/g 1
D52242.2	MP	p/g	CD I	p/g 1	p/g 1
D5412.1	MP	p/g	CD II	p/g 2	p/g 2
D5412.2	MP	p/g	CD II	p/g 2	p/g 2
D5413.1	MP	p/g	CD II	p/g 2	p/g 2
D5432.1	MP	p/g	CD II	p/g 2	p/g 2
D5432.2	MP	p/g	CD I	p/g 1	p/g 1
D5433.2	MP	p/g	CD I	p/g 1	p/g 2
E1122.1	MP	p/g	EH II	p/g 1	p/g 2
E1122.2	MP	p/g	EH I		p/g 2
E1123.1	MP	p/g	EH II	p/g 1	p/g 2
E1123.2	MP	p/g	EH I	p/g 1	p/g 1
E1131.1	MP	p/g	EH I	p/g 1	p/g 2
E1132.1	MP	p/g	EH II	p/g 2	p/g 2
E1522.1	MP	p/g	EH II	p/g 2	p/g 2
E1533.1	MP	p/g	EH I	p/g 1	p/g 1
E1533.2	MP	p/g	EH I	p/g 1	p/g 1
E5223.1	MP	p/g	EH II	p/g 1	p/g 2
E5223.2	MP	p/g	EH I	p/g 1	p/g 1
E5232.1	MP	p/g	EH I	p/g 1	p/g 2
E5232.2	MP	p/g	EH II	p/g 2	p/g 2
E5233.1	MP	p/g	EH II	p/g 1	p/g 2
E5311.1	MP	p/g	EH I	p/g 1	p/g 2
E5311.2	MP	p/g	EH I	p/g 1	p/g 1
E5312.1	MP	p/g	EH II	p/g 1	p/g 2
E5312.2	MP	p/g	EH II	p/g 2	p/g 2
E5322.1	MP	p/g	EH I	p/g 1	p/g 1

E5322.2	MP	p/g	EH I	p/g 1	p/g 1
E5323.1	MP	p/g	EH I	p/g 1	p/g 1
E5323.2	MP	p/g	EH I	p/g 1	p/g 1
H1113.2	MP	p/g	EH II	p/g 2	p/g 2
H1511.1	MP	p/g	EH I	p/g 1	p/g 1
H1511.2	MP	p/g	EH II	p/g 2	p/g 2
H1532.1	MP	p/g	EH II	p/g 2	p/g 2
H1533.1	MP	p/g	EH I	p/g 1	p/g 1
H1533.2	MP	p/g	EH I	p/g 1	p/g 1
H2212.2	MP	p/g	EH II	p/g 1	p/g 2
H2222.1	MP	p/g	EH II	p/g 2	p/g 2
H2222.2	MP	p/g	EH I	p/g 2	p/g 2
H2223.2	MP	p/g	EH II	p/g 1	p/g 2
H2411.1	MP	p/g	EH II	p/g 2	p/g 2
H2413.1	MP	p/g	EH I	p/g 2	p/g 2
H2413.2	MP	p/g	EH I	p/g 2	p/g 2
H2431.1	MP	p/g	EH I	p/g 1	p/g 1
H2431.2	MP	p/g	EH I	p/g 1	p/g 2
H2432.1	MP	p/g	EH I	p/g 1	p/g 1
H2432.2	MP	p/g	EH II	p/g 2	p/g 2
H2511.2	MP	p/g	EH I	p/g 1	p/g 1
H2513.1	MP	p/g	EH II		p/g 2
H2513.2	MP	p/g	EH II	p/g 2	p/g 2
H2521.1	MP	p/g	EH II	p/g 1	p/g 2
H2523.1	MP	p/g	EH II	p/g 2	p/g 2
H2523.2	MP	p/g	EH I	p/g 1	p/g 1

Table 3.2
List of plants regenerated
after storage regimes

Plant name	Type	Tissue for DNA analysis	Filter name	Tubers for protein analysis	Tubers for field trial
A.D6	CST	i/v	CST	--	i/v
A.L12	CST	i/v	CST		i/v
B.L6	CST	i/v	CST		i/v
B.D6	CST	i/v	CST	p/g 1	i/v
B.L12	CST	i/v	CST	p/g 1	i/v
C.L6	CST	i/v	CST	p/g 1	i/v
C.D6	CST	i/v	CST		i/v
C.L12	CST	i/v	CST	p/g 1	i/v
D.L6	CST	i/v	CST		i/v
D.D6	CST	i/v	CST	p/g 1	i/v
D.L12	CST	i/v	CST		i/v
E.L6	CST	i/v	CST		i/v
E.D6	CST	i/v	CST		i/v
E.L12	CST	i/v	CST		i/v
H.L6	CST	i/v	CST	p/g 1	i/v
H.D6	CST	i/v	CST		i/v
H.L12	CST	i/v	CST		i/v
SA2	OST	i/v	OST	p/g 1	p/g 1
SA4	OST	i/v	OST	p/g 1	p/g 1
SB1	OST	i/v	OST	p/g 1	p/g 1
SB2	OST	i/v	OST	p/g 1	p/g 1
SB4	OST	i/v	OST	p/g 1	p/g 1
SC2	OST	i/v	OST	p/g 1	p/g 1
SD1	OST	i/v	OST	p/g 1	p/g 1
SD2	OST	i/v	OST	p/g 1	p/g 1
SD4	OST	i/v	OST	p/g 1	p/g 1
SE1	OST	i/v	OST	p/g 1	p/g 1
SE2	OST	i/v	OST	p/g 1	p/g 1
SE3	OST	i/v	OST	p/g 1	p/g 1
SH1	OST	i/v	OST	p/g 1	p/g 1
SH2	OST	i/v	OST	p/g 1	p/g 1
SH3	OST	i/v	OST	p/g 1	p/g 1
SH4	OST	i/v	OST	p/g 1	p/g 1

Table 3.3
List of plants regenerated
from leaf-disc callus

Plant name	Type	Tissue for DNA analysis	Filter name	Tubers for protein analysis	Tubers for field trial
VA1	REG	p/g	V() I	p/g 2	p/g 2
VA3	REG	p/g	V() I	p/g 2	p/g 2
VA4	REG	p/g	V() I	p/g 2	p/g 2
VA5	REG	p/g	V() I	p/g 2	p/g 2
VA8	REG	p/g	V() I	p/g 2	p/g 2
VA9	REG	p/g	V() I	p/g 2	p/g 2
VA10	REG	p/g	V() I	p/g 2	p/g 2
VB1	REG	p/g	V() I	p/g 2	p/g 2
VB2	REG	p/g	V() I	p/g 2	p/g 2
VB4	REG	p/g	V() I	p/g 2	p/g 2
VB5	REG	p/g	V() I	p/g 2	p/g 2
VB7	REG	p/g	V() I	p/g 2	p/g 2
VB8	REG	p/g	V() I	p/g 2	p/g 2
VB9	REG	p/g	V() I	--	--
VB10	REG	p/g	V() I	p/g 2	p/g 2
VC1	REG	p/g	V() I	--	--
VC3	REG	p/g	V() I	p/g 2	p/g 2
VC4	REG	p/g	V() I	p/g 2	p/g 2
VC5	REG	p/g	V() I	p/g 2	p/g 2
VC6	REG	p/g	V() I	p/g 2	p/g 2
VC7	REG	p/g	V() I	--	p/g 2
VC8	REG	p/g	V() I	--	--
VC9	REG	p/g	V() I	--	--
VC10	REG	p/g	V() I	--	p/g 2
VD1	REG	p/g	V()II	p/g 2	p/g 2
VD2	REG	p/g	V()II	p/g 2	p/g 2
VD3	REG	p/g	V()II	--	--
VD6	REG	p/g	V()II	p/g 2	p/g 2
VD7	REG	p/g	V()II	p/g 2	p/g 2
VE1	REG	p/g	V()II	p/g 2	p/g 2
VE2	REG	p/g	V()II	p/g 2	--
VE3	REG	p/g	V()II	p/g 2	p/g 2
VE5	REG	p/g	V()II	p/g 2	p/g 2
VE6	REG	p/g	V()II	p/g 2	p/g 2
VE8	REG	p/g	V()II	p/g 2	p/g 2
VE10	REG	p/g	V()II	p/g 2	p/g 2
VH1	REG	p/g	V()II	p/g 2	p/g 2
VH2	REG	p/g	V()II	p/g 2	p/g 2
VH3	REG	p/g	V()II	p/g 2	p/g 2
VH4	REG	p/g	V()II	--	--
VH5	REG	p/g	V()II	--	--
VH6	REG	p/g	V()II	--	--
VH7	REG	p/g	V()II	p/g 2	p/g 2
VH8	REG	p/g	V()II	p/g 2	p/g 2
VH10	REG	p/g	V()II	p/g 2	p/g 2
VHX	REG	p/g	V()II	--	--
VC2	REG	--		p/g 2	p/g 2
VD5	REG	--		p/g 2	p/g 2
VE4	REG	--		p/g 2	p/g 2

VE9	REG	--	p/g 2	p/g 2
VHY	REG	--	p/g 2	p/g 2
VX1	REG	--	p/g 2	p/g 2
VX2	REG	--	p/g 2	p/g 2
VX3	REG	--	p/g 2	p/g 2
VX4	REG	--	p/g 2	p/g 2

Chapter 4. Development of restriction fragment analysis for assessment of stability

4.1 Introduction

Restriction fragment analysis of DNA involves various techniques which are the backbone of modern molecular biology. Although the basic techniques are now standard (see Maniatis et al 1982), modifications are being developed rapidly to take advantage of improvements in equipment and materials. Thus, as a range of methods have been developed, these can be tailored to the specific application. In this study the requirement was for rapid, simple and, preferably, cheap methods that could be used in a routine manner to screen many plants.

A summary of the analysis is shown in Figure 4.1. Genomic DNA is digested with a restriction endonuclease enzyme to yield fragments whose size depends on the sequence of DNA. These are separated (on the basis of size) by gel electrophoresis, denatured (to allow hybridisation to probe DNA later) and transferred to a solid membrane or 'filter'. After baking to fix the genomic DNA fragments permanently to the filter, the fragments on the filter are hybridised to labelled DNA of a specific sequence (the 'probe'). The filter is washed after this to remove probe that does not hybridise specifically to homologous DNA and then the probe is visualised (in this case by autoradiography as the probe is radioactively labelled).

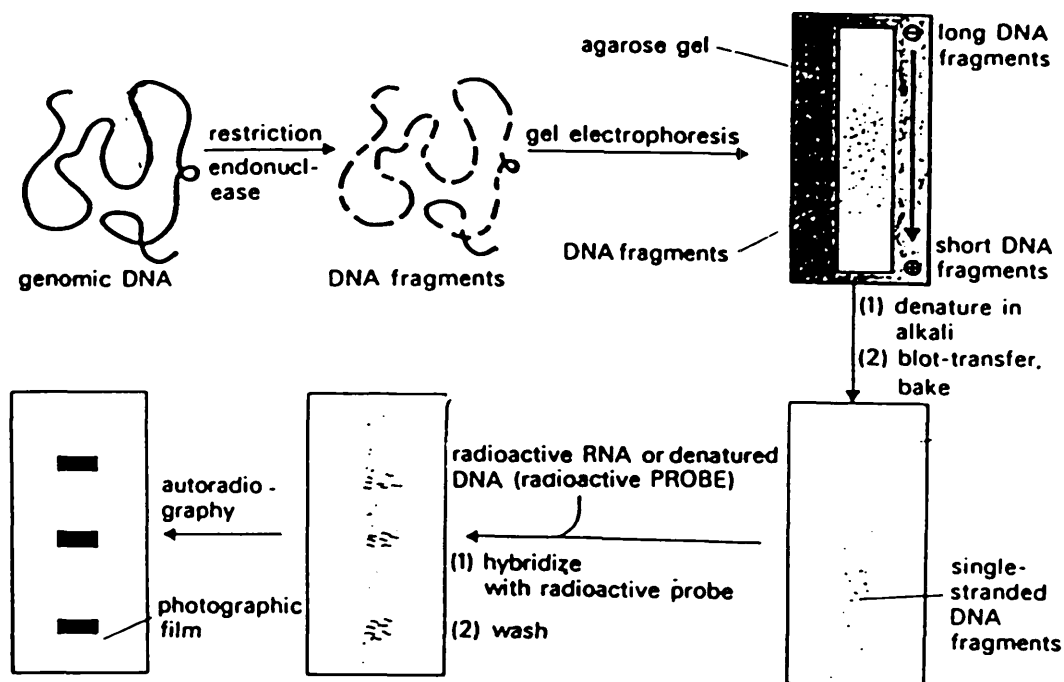


Figure 4.1. Summary of restriction fragment analysis
See text for details and explanation.

As the hybridisation of DNA is highly sequence specific, the areas of the filter shown up as dark bands on the autoradiograph will contain genomic DNA homologous to the probe sequence, of a size determined by the genomic DNA sequence homologous to the probe (as a result of digestion by the restriction endonuclease). Alterations in the sequence of DNA in regions which affect the restriction endonuclease recognition sites (a short sequence of bases) relative to each other will thus alter the size, and therefore the position on the filter, of the DNA homologous to the probe - resulting in the band being visualised in a different place.

This is illustrated in Figure 4.2. A shows a piece of

genomic DNA with its recognition sites for two different restriction enzymes marked by arrows. The band pattern produced after appropriate restriction enzyme digestion, other manipulations and hybridisation with the indicated probe, is shown below - for both enzymes, 3 bands for EcoRI and 2 for HindIII.

B is the same piece of genomic DNA but with a deletion. This has the effect of shortening the size of some of the fragments - shown below by bands with higher mobility further down the gel. C shows the genomic DNA with a point mutation that destroys a recognition site for EcoRI. This is revealed as one larger band - the sum of two smaller bands. The HindIII pattern is not altered by this mutation.

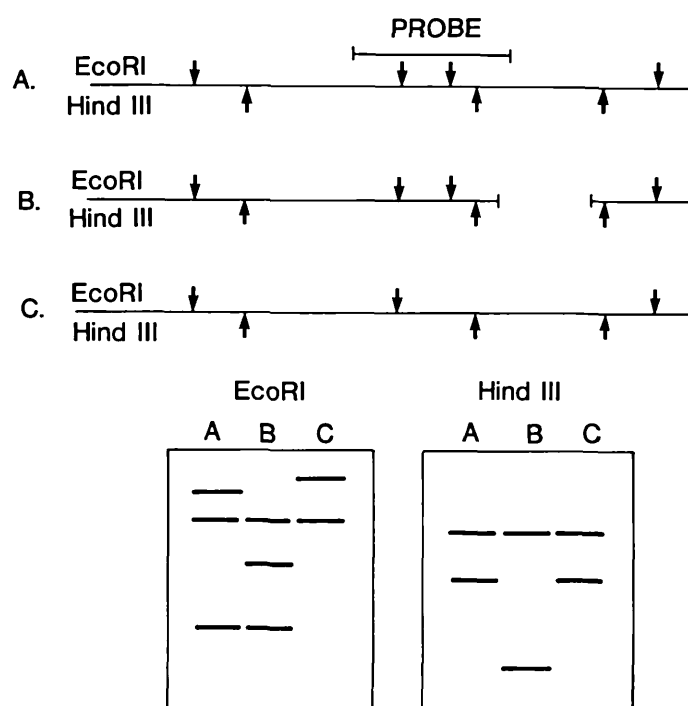


Figure 4.2 Generation of restriction fragment size alterations
Schematic representation of fragments of DNA and the band pattern produced by restriction with two different enzymes and hybridisation with the probe shown. See text for explanation.

Two points to bear in mind from this are: (i) that the probe DNA - often a very small piece of DNA - hybridises to (and can therefore show mutations in) much larger genomic fragments, and (ii) some mutations only affect the band pattern with respect to one enzyme - the DNA appears to be identical if a different restriction enzyme is used.

This shows how restriction fragment length alterations can occur. Any alteration in DNA which changes either a restriction enzyme recognition site, or the position of such sites relative to one another, has the potential to be visualised by these techniques. It is probable that genetic instability in culture (somaclonal variation) involves such changes. Thus it is valid to use such techniques to find evidence for somaclonal variation and to check the stability of micropropagated stocks.

The experimental scheme for the application of restriction fragment analysis to the study of plants after specific *in vitro* treatments is shown in Figure 4.3. The first box - the production of plant material - has been described in Chapter 3. The analysis of the bands and the subsequent assessment of stability will be presented in Chapter 5. In this chapter the development of the following methods is described: (i) DNA isolation and purification, (ii) DNA restriction, fragment separation, and blotting (iii) probe isolation and labelling and (iv) hybridisation. The development of the techniques for the specific application

of this study and some other of the modifications relevant to this work are also described

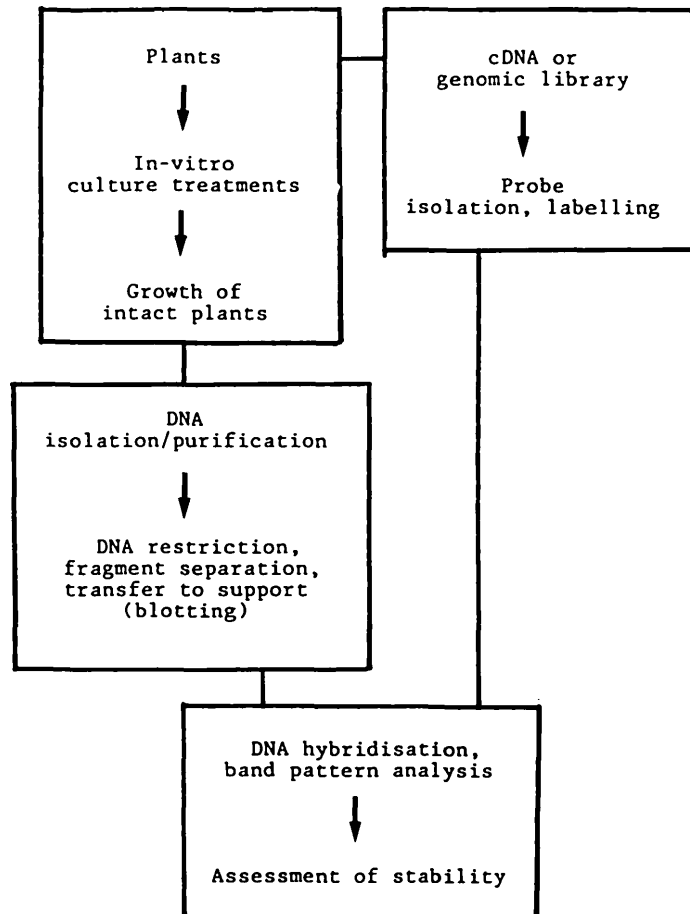


Figure 4.3 Application of restriction fragment analysis to the study of plants after *in vitro* treatments
See text for details

4.2 DNA Isolation/purification

Very pure samples of DNA can routinely be obtained by using caesium chloride gradients (Maniatis et al 1982), but not only are these very time consuming and laborious to obtain, but also quite expensive both in the equipment required and in the consumable costs. Thus a requirement for this work was the identification or development of a suitable rapid, cheap and reliable method of DNA isolation. In this respect, absolute purity was not the overriding concern - rather that the DNA should be of sufficient purity for restriction enzyme digestion and electrophoresis.

Because of the cellulose cell wall of plant cells, almost all genomic DNA extractions start with grinding the tissue - often in liquid nitrogen. The ground tissue is then suspended in buffers containing EDTA (to chelate divalent metal ions - especially magnesium which is required by DNA degrading enzymes), sodium chloride and SDS, and this is usually followed by extraction with phenol and chloroform. Alcohol precipitations and further phenol/chloroform extractions are often the only purification steps needed.

One such method (Plant genomic DNA extraction I) developed by Dr. B D Hall was initially used for this study. However large amounts of some impurities in the final samples made the DNA difficult to resuspend and so interfered with both restriction enzyme digestion and gel electrophoresis. Further work may have led to an improvement of this method - a dark treatment to deplete the leaf tissue of starch prior to

extraction was suggested - but a quite different method of extraction was discovered which proved to be superior, and was subsequently adopted.

This method (Plant genomic DNA extraction II) has been modified only slightly from that of Dellaporta et al (1985) in that it was found useful to incorporate an RNase treatment in the extraction procedure, rather than combining RNase treatment with restriction enzyme digestion later. As well as the much cleaner DNA produced as a result of this protocol, the DNA also appeared to be composed of molecules with slightly larger average size (Figure 4.4 a & b). This was attributed to the absence of phenol/chloroform extractions, which, to be efficient, require quite vigorous mixing of the two phases - causing shearing of the high molecular weight DNA molecules.

4.3 Restriction, Electrophoresis, Blotting

These three subjects have been grouped together in this section as the methods chosen for each step often depend upon which method has been employed for previous steps.

Restriction endonucleases are enzymes which digest DNA. There are three types: I, II & III - the most useful being type II enzymes which recognise a specific base sequence and cleave the DNA at that point. The other types of endonuclease show less specificity in where they cleave the DNA, and as it is this specificity (with lengths of restriction fragments being thus dependent on DNA sequence) which

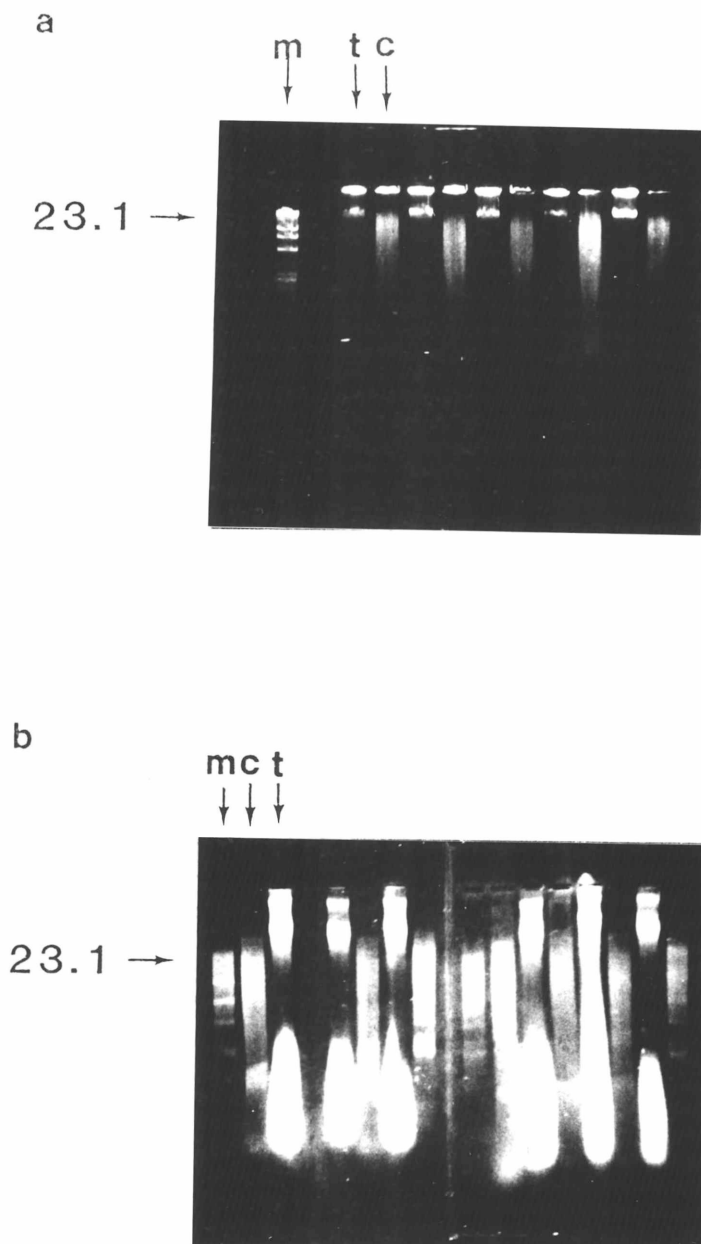


Figure 4.4 Comparison of extracted DNA samples

Total (t) and restricted (c) DNA from the two different extraction protocols, a is from DNA extraction I and b from DNA extraction II. m is HindIII restricted lambda DNA as marker. Note the greater size of the total DNA in b (mostly above the 23.1 kb lambda band) and the much better restriction with no DNA left in the well - as compared with a. The large smear lower down the gel in the total tracks of b is RNA, not removed in this particular extraction. An RNase step was incorporated to remove this during the extraction procedure.

is vital to restriction fragment analysis, types I and III are seldom used.

Many different enzymes are now available commercially that recognise different base sequences. As nothing is known about whether somaclonal variation is base sequence specific, the choice of enzyme is purely arbitrary - often being made on the basis of cost and availability. However it can be seen that enzymes recognising different length sequences (sequences of four or six bases are most common) will produce fragments with significantly different average lengths, because the recognition site will occur much more often for the four base enzymes than for the six base enzymes. This difference in average size of the fragments means that different separation conditions are required to separate the fragments produced by these different classes of enzymes, and so the separation system employed is often chosen to complement the class of restriction enzyme used.

For this study 'six-cutters' (restriction enzymes that recognise a six base pair sequence) were chosen and used exclusively. Although it would have been useful to compare the class of enzyme used, it was felt more productive results would be obtained by keeping the methodology of restriction fragment analysis constant, and using 'four-cutters' as well as 'six-cutters' would involve developing another separation protocol.

Six-cutters were chosen (rather than four) for a number of reasons. First, a wide range are available covering very different recognition sequences and they are, relatively, cheap. Second, any six base pair palindromic sequence would be expected to occur every 4,096 base pairs (on average) putting the vast majority of restriction fragments within the size range 800 bp (base pairs) to 20 kbp (kilo-base pairs). With the production of larger fragments a bigger proportion of the genome can be analysed with each probe used, which means that there is a greater chance of detecting insertions and deletions in the DNA. Using these enzymes does reduce the chance of detecting point mutations (as less of the genome is sampled at the base sequence level) but this is hardly increased by using four-cutters because although they cut every 256 bp (on average) only four bases at each site are analysed rather than six.

There is evidence that use of this system is the most likely to reveal variation. McCouch et al (1988) and Landry et al 1987 identifying restriction fragment polymorphisms in rice and lettuce, respectively, found that six-cutters were much better than four-cutters and that, in general, the longer the average size of hybridising fragment, the more likely it was to detect polymorphism. This implies that that the polymorphisms were due to insertions or deletions of DNA sequences and not to point mutations (at least the polymorphisms detected were - there may have been as many point mutations but they were not revealed by this analysis). Evidence that somaclonal (or tissue culture induced) variation does not consist solely of point mutations has come from

studies in which somaclonal variation was compared with classical mutagenesis using chemical mutagens (Gavazzi et al 1987). This showed that the spectrum of changes caused by the two different treatments was qualitatively different. That is, the two treatments caused different phenotypic mutations, and as the chemical mutagenesis is known to cause single base changes, it suggests that somaclonal variation is not caused by the same mechanism.

Using the same rationale, enzymes that recognise longer sequences would be even better, but here the problems of resolving the fragments overcome the advantages. Reversed-field electrophoresis can be used to resolve very big fragments of DNA (millions of base pairs) (reviewed by Knight 1989) but only correspondingly large insertions or deletions can be detected. Also very few enzymes which cut at larger sites are known and so the analysis would be incomplete.

The resolution of restriction fragments by gel electrophoresis is clearly of central importance to the analysis of these fragments. As mentioned above, the system of electrophoresis chosen depends on the size of fragments to be separated and the resolution required. By deciding on one particular approach (using six-cutters) to produce the fragments, one system of electrophoresis can be used throughout with the conditions being optimised and then kept constant.

For most DNA analysis, agarose gels are commonly used at 0.5 to 2.0 %, which gives separation of quite large fragments

(50kbp to 100kbp depending on the particular concentration) and resolution down to about 50bp (again depending on the particular concentration used). Acrylamide gels cannot be handled below a concentration of 3 % but can be made up at much higher concentrations, so enabling resolution of single base size differences for sequencing applications.

In this study, agarose gels at a concentration of 0.75 % were used as this gave good separation of fragments in the range 20kbp to 500bp. Gels were 25 cm long - the longest that could be obtained using the equipment available - to enable the best separation (and so resolution of smaller size differences between fragments), while not losing smaller fragments. The blotting and hybridisation system available (see later) allowed a maximum size of gel of 20 x 20 cm for further processing. This was achieved by removing the top 5 cm of gel after electrophoresis - this portion contained only the wells with almost no DNA fragments. After restriction enzyme digestion, very few fragments larger than 20kbp were present and this top 5 cm of gel only contained fragments above 30kbp (as determined by molecular size markers). This is illustrated in Figure 4.5.

Gels were prepared and run in Tris borate buffer, which has a fairly high buffering capacity to prevent any problems of buffer exhaustion during the relatively long run used for these gels. The gels were run at 50 mA for 13 hours (+/- 30 minutes). This resulted in loss of fragments smaller than 1kbp. However the price of retention of such fragments was loss of resolution of size differences in the larger

fragments, and so it was felt that this was unavoidable. As mentioned previously, analysis of the larger fragments is

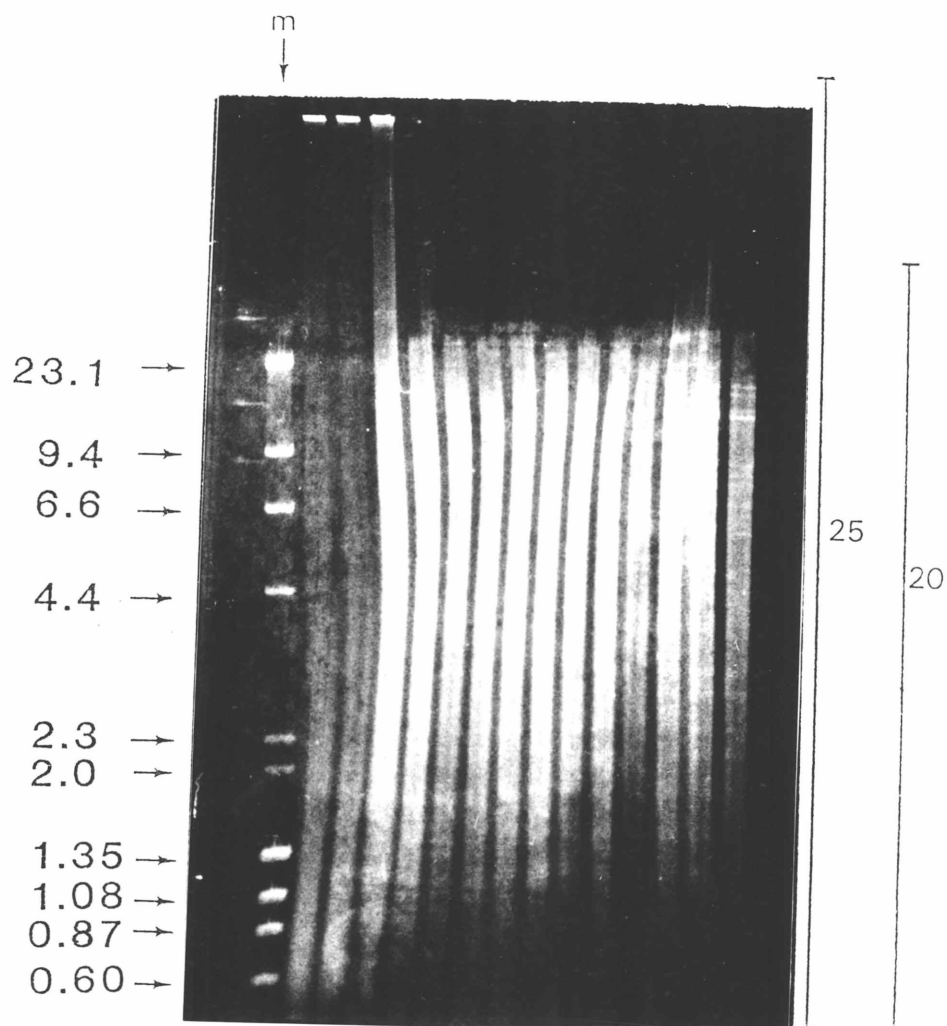


Figure 4.5 Gel of restricted DNA samples

The two size bars show the original size of the gel, and the size of the trimmed gel used for blotting. *m* is the marker lane containing DNA size markers. Note bands superimposed on the smear, especially in the lighter lanes. These are probably organellar DNA and are indicative of complete restriction.

more likely to reveal mutation, and so these larger fragments are relatively more important than the smaller fragments. By keeping the volume of gel constant at 200 ml and the volume of buffer in the tank constant at 1500 ml,

the conditions were kept as similar as possible for all the gels produced.

Initially, for the development of the system and the early screening of regenerant plants to check that variation could be found, combs were used which had 15 teeth 8 mm wide. For later analysis, in order to analyse more plants at one time, combs with 30 teeth were used, which allow twice the number of samples on each gel and so reduced the number of gels (and subsequent filters) required by half (Figure 4.6). Using the latter comb did not produce bands as sharp as those produced with the previous comb, but a second advantage was that smaller amounts of restricted DNA could be used - 3-5 μg rather than 7-10 μg .

The absolute amount of DNA loaded and run on the gel is not critical provided that it is kept within certain limits. In order to identify low copy number sequences on subsequent hybridisation a certain minimum amount must be loaded. At the same time, loading too much DNA can cause smearing of bands because fragments interfere with each other during electrophoresis. Because potato has a relatively small amount of DNA (about 4 pg DNA per haploid genome) the low copy number probes used in this study could be detected in as little as 5 μg of restricted genomic DNA loaded into the larger well (the 15 tooth comb). 10 μg loaded into the same well did not produce any smearing - even with higher copy number probes (Figure 4.7). Thus for the larger comb 7-10 μg was considered optimum. When the smaller comb (30 teeth) was used, optimisation studies were not carried out, because it was considered adequate to simply reduce the amount of

DNA loaded by half - since the well volume was reduced by this factor. Further analysis showed this to be adequate.

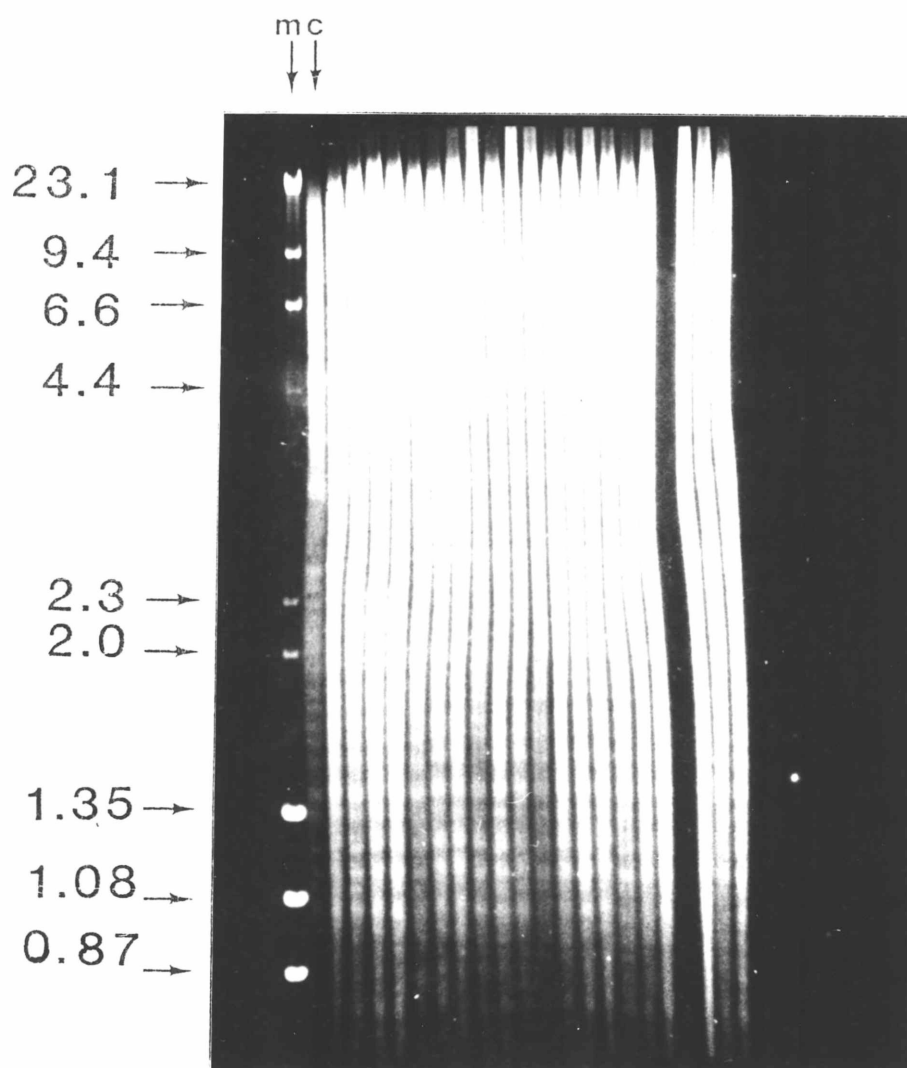


Figure 4.6 Gel used for main analysis

This gel was produced with a 30 tooth comb, and half of the amount of DNA used previously. Note that the organellar bands can now not be seen. m is the marker lane with sizes are in kilobases, c is the control DNA lane.

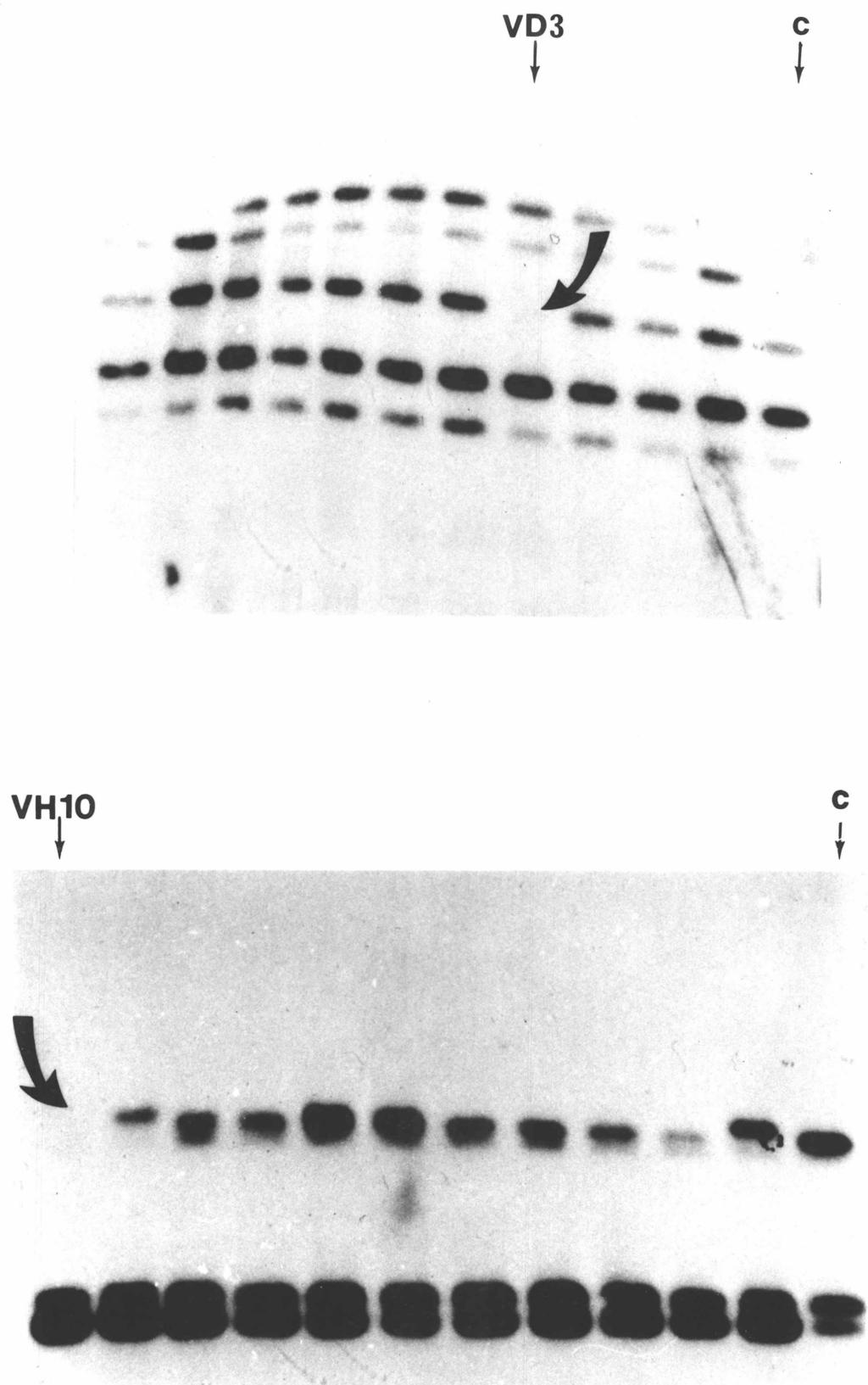


Figure 4.7 Two autoradiograms with different copy number probes

These two autoradiograms are of the same filter, but probed with different probes. Top is the low copy number probe SS15, and bottom is the repetitive probe 4324 (see Chapter 5). Note that, although the bands are clearly seen with the lowcopy number probe, no smearing is observed with the repetitive probe.

These also show examples of variant band patterns (arrowed). Plant VD3 (top) has a band completely missing, compared with the control lane c. Plant VH10 (bottom) also appears to have a band missing, but really this is actually a reduction in copy number, shown if a longer exposure is carried out, when the band can be seen.

Because the DNA extraction procedure used did not produce DNA pure enough to quantify by the standard spectrophotometric absorption techniques, the amount of DNA was estimated by comparison of the fluorescence of bound ethidium bromide in which gels were stained to visualise the DNA. Aliquots from the restriction reactions were run on smaller gels to assess the completion of restriction (estimated by the absence of high molecular weight bands and also higher intensity bands from satellite or organellar DNA superimposed on the smear)(Figure 4.8). These gels also gave an estimate of the amount of DNA by comparison with standards (sheared herring sperm DNA). Although this is only a rough guide, it is sufficiently accurate and makes possible even loading of tracks (that is loading of tracks with similar amounts of DNA).

Molecular weight markers were included on each gel to give a record and enable size estimates of hybridising bands to be produced. A mixture of two commercial preparations was used (see Table 4.1) which gave fragments ranging from 23.5kbp to 800bp (in fact other smaller fragments were also present - but these were run off during electrophoresis). These were visualised after electrophoresis by staining with ethidium bromide and illumination with UV. The gels were then photographed to provide a record which was later used to line up the size markers on the autoradiographs of the hybridised filters. The size markers do not appear on the autoradiograph as the probes do not hybridise to these sequences.

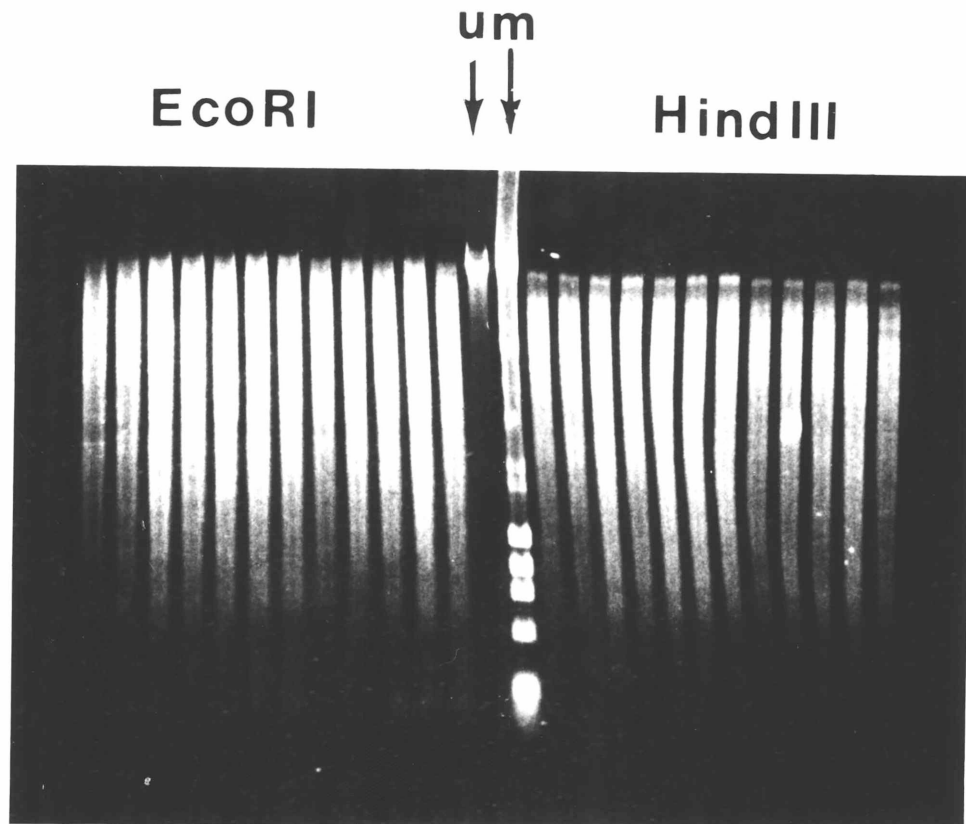


Figure 4.8 Aliquots from restriction reactions

These gels were run to assess the amount of DNA in each sample as well as to ensure complete restriction. DNA from the same plants was cut with the two enzymes and then run together for a further comparison. Note the different appearance of DNA restricted by the two different enzymes, these are typical for the enzymes used. Organellar bands can just be seen superimposed on the smear of genomic DNA, indicating complete digestion. Note also the lower amount of DNA in the two leftmost lanes (both enzymes). These will be resuspended in a smaller final volume in order to equalise DNA concentration and enable comparison on the autoradiographs. m is the molecular size marker, and u is uncut DNA to show size before restriction.

Transfer of DNA (blotting) is inefficient with very large fragments. For this reason the fragments in the gel are nicked after electrophoresis to aid equal transfer of all size classes. Depurination in hydrochloric acid is widely used, but for this study illumination with short wave UV was used as the simplest method.

HindIII restricted lambda DNA	HaeIII restricted øx174 DNA
23.1	
9.4	
6.6	
4.4	
2.3	
2.0	
	1.35
	1.08
	0.87
	0.60
0.56	
	0.31
	0.28
	0.27
	0.23
	0.19
	0.12
	0.07

Table 4.1 DNA size markers

Marker lanes contained a mixture of these two commercial preparations, which have bands of the sizes above, in kilobases. Only those bands above the line are visible on most gels.

In order to allow hybridisation to homologous probe sequences, the DNA in the gel must be made single-stranded (or denatured). This is accomplished by soaking the gel in sodium hydroxide solution for one hour, usually with shaking to ensure even diffusion of the sodium hydroxide throughout the gel. Because the membrane used to produce the filter is only positively charged (and thus able to bind the negatively charged DNA) at neutral or acid pH, the gel must then be neutralised before transfer takes place. Sodium acetate was used as the buffer recommended by the suppliers of the membrane.

Many new membranes are now positively charged at all pHs and can even bind double stranded DNA, thus making it possible to transfer in alkaline buffers or to denature DNA on the

filter itself (Khandjian 1987). However these were not available at the start of this study and since conditions were initially optimised with the pH sensitive membrane it was kept constant throughout. The membrane used was Biodyne A (Pall) a nylon membrane which itself has advantages over the original nitrocellulose. Its strength and durability mean that repeated rounds of hybridisation can be performed on the same filter - thus reducing the number of filters that need to be prepared.

The original Southern blotting approach utilised capillary action to draw solution through the gel and membrane, transferring the DNA onto the filter (Southern 1975). This was prone to problems associated with maintaining an even flow of solution to ensure that transfer was accomplished over the whole filter, retaining the orientation of the fragments relative to one another. Care had to be taken that contact was not made between the blotting stack and the solution reservoir (or the gel itself for that matter) outside the filter. As the resistance to flow through the filter was greater, such contact would reduce this flow and so the transfer would be affected. Because of these problems, and the requirement for a reliable, repeatable system, another method of transfer was evaluated - using electric current.

This involved sandwiching the gel and filter between two electrodes in a tank with buffer and passing an electric current through the sandwich. As the resistance of the gel and filter is the same as that of the buffer (gel and filter are equilibrated with buffer before setting up), current

flow and hence transfer of DNA is even and fairly rapid (4 - 5 hours rather than 16 - 18 for capillary blotting). Using a commercially available system, (Biorad Transblot Cell) two gels could be transferred at the same time. This system was adopted early on in the study and was subsequently used throughout, utilising the conditions recommended by the filter suppliers (Pall 1987). After transfer the DNA is bound to the filter by drying - with this particular membrane baking in an air oven at 80°C for one hour is required. The dry filters are stable and can be stored in plastic bags at room temperature until used for hybridisation.

4.4 Probe Isolation/Labelling

The best source of probes for restriction fragment analysis depends to a large extent on which organism is being studied, as well as on the type of study and its requirements. To identify polymorphisms for RFLP mapping the early work done on mammalian genomes used probes isolated from genomic libraries. The much larger proportion of repetitive DNA in plant genomes means that this approach yields many highly repetitive probes of little use for detecting polymorphism. cDNA libraries are thus better sources because many more probes represent single or low copy number sequences (Helentjaris et al 1985, 1986). Other approaches involve enriching genomic libraries for low copy sequences (or repetitive if required) (eg. McCouch et al 1988) or screening libraries for the desired type of probe before isolation and use (eg. Landsmann and Uhrig 1985).

Because this study is not directed towards the detection of polymorphic loci between genetically distinct individuals, but is concerned rather with the detection of tissue culture induced mutation in a population of clonally produced plants, the requirements are somewhat different. Low or single copy number clones would be very sensitive - revealing small insertions, deletions and even some point mutations, but the proportion of the genome analysed with each probe would be very small. Repetitive clones would, on the other hand, allow analysis of large portions of the genome at one time, but the sensitivity to detect small or subtle alterations would be lost. Only large alterations in copy number would be revealed while changes that alter restriction fragment length in only one locus are unlikely to be detected amongst the much stronger signal from the other loci.

A possible middle line could be steered by selecting low copy repeated sequences or coding sequences from multiple gene families. Molecular evidence for somaclonal variation in potato protoclones was found using a low-to-middle copy number repetitive sequence isolated by screening a genomic library. This was achieved by probing the library with labelled total genomic DNA and isolating weakly hybridising clones - strongly hybridising clones would correspond to highly repetitive clones while no signal would suggest low or single copy sequences (Landsmann & Uhrig 1985). This clone was subsequently shown to contain ribosomal DNA sequences and was obtained for this study.

Because it was not known what form the molecular evidence for somaclonal variation would take, it was decided to use a range of different types of probe sequences and to study which would be most useful for future analyses. The ribosomal probe (obtained from Dr H Uhrig MPI Cologne, mentioned above), clone pGMO1 (Mignery et al 1984) - a patatin cDNA clone as an example of a multi-gene family (originally from Dr W Park Texas), and two putative single copy clones (isolated from a cDNA library of potato prepared at Rothamsted by Dr M Burrell) have been used to probe filters with DNA of all the plants produced.

The isolation of DNA to use as probe can be achieved by many methods, depending on the vector used to clone the DNA and also on the purity required. In all cases the requirement is for extra-chromosomal DNA and so lysing the bacteria is done very carefully to prevent release of the bacterial chromosomal DNA. Caesium chloride density gradient centrifugation will separate closed circular DNA (plasmid) from linear DNA (chromosomal) but this is costly and time-consuming and can usually be avoided, especially for small volume preparations which suffer from fewer problems with chromosomal DNA contamination.

Plasmid DNA extraction method I (Birnboim and Doly 1979), was used here for a relatively small preparation, but can be used for large scale plasmid isolation and yields DNA of high purity - good enough for all other subsequent manipulations. Labelling reactions, however, do not necessarily need DNA of such high purity, and, as they only

require small amounts of DNA as substrate, small 'mini-prep' isolation protocols are sufficient, for example Plasmid DNA extraction II . The probes used here were all isolated by one or the other of these two methods.

Providing that there is no homology between the plasmid vector sequence and the genomic DNA being analysed, the whole plasmid can be labelled and used as a probe. However, as this results in a reduction in the amount of probe DNA that is homologous to the target (the genomic DNA on the filter), this reduces the efficiency of hybridisation. Because of this, the insert (the piece of DNA cloned into the plasmid) is nearly always removed from the plasmid and labelled separately to use as probe. An added advantage here is that it removes any possibility of background hybridisation or artifacts being due to cross hybridisation of vector with target DNA.

The insert is removed by digestion with the appropriate restriction enzyme(s) and separation is by electrophoresis through gels. Acrylamide gels are useful if the insert and vector are very similar in size (acrylamide gels provide the best resolution) but agarose gels are nearly always sufficient (Figure 4.9). Purification of the insert from the gel can cause problems because electro-elution from agarose also releases sulphated polysaccharides, which may interfere with the labelling. Use of higher purity agarose solves this problem, but new labelling techniques now remove the need for purification altogether.

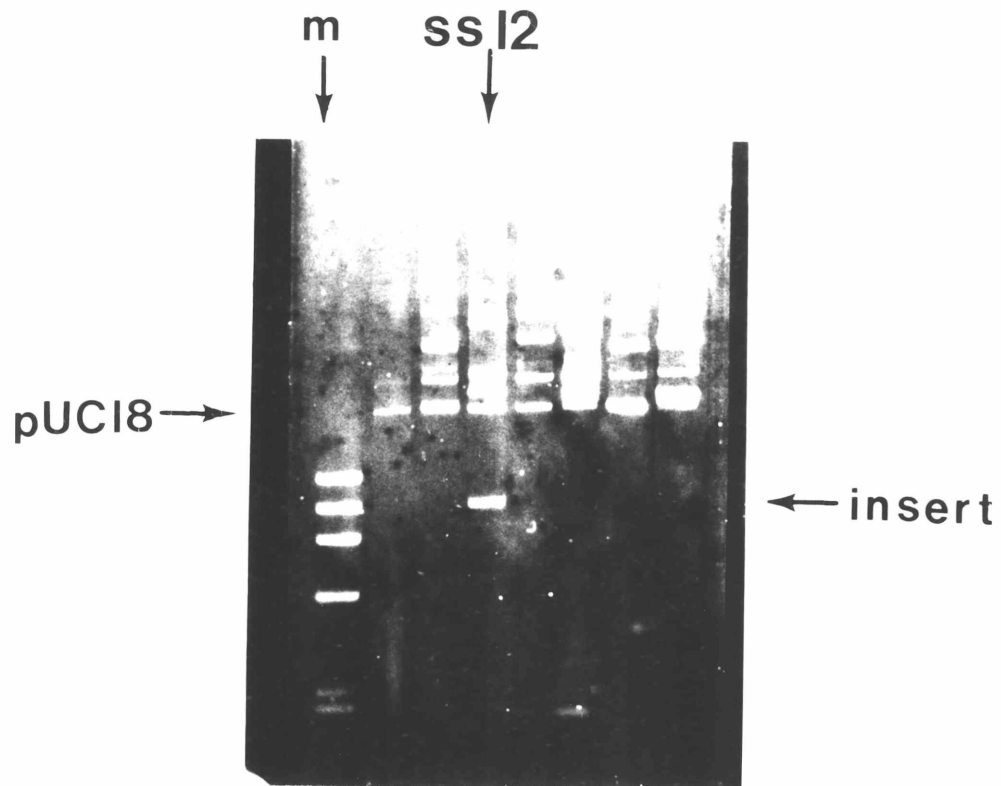


Figure 4.9 Separation of insert from vector plasmid

Restricted plasmids run on a low gelling temperature agarose gel in order to separate the insert from the vector. Restricted vector (in this case pUC18) is also run with the plasmids, to distinguish inserts from the vector DNA. m is HaeIII restricted ϕ x174 DNA which indicates the size of SS12 to be $\approx$ 1 kilobase. The portion of gel containing the insert is removed from the gel and solubilised to release the DNA for labelling and subsequent use as a probe.

The original labelling technique of 'nick-translation' (Rigby et al 1977) required two enzymes; DNase I to produce single-strand nicks in the DNA, and DNA Polymerase I to synthesise new DNA (incorporating the labelled nucleotide) as it degraded the old strand, from the nick onwards. These two enzymes were very sensitive and could be inhibited by impurities in the mixture, and the whole reaction was inefficient because with time the polymerase starts to degrade

the already labelled strand of DNA.

'Oligo-labelling' (Feinberg & Vogelstein 1983) works with single-stranded (denatured) DNA, using the large fragment of DNA Polymerase I (Klenow fragment) to synthesise a new labelled strand with random hexa-nucleotides as primers. The Klenow fragment has the 5' - 3' polymerase activity and the 3' - 5' exonuclease activity (for proofreading) but not the 5' - 3' exonuclease activity of the holoenzyme (Old and Primrose 1985). This means that it cannot degrade a strand as it moves along, thus preventing digestion of previously labelled DNA. As a result, the incorporation of label by this method is much higher, reaching up to 80 % of the labelled nucleotide added, and the specific activity of the labelled DNA is higher, which gives more sensitive detection of bands on the filter. Also the reaction can be allowed to proceed for much longer without reducing the size of labelled fragments, which happens in 'nick-translation' because the DNase enzyme is continuously making new nicks in the DNA. The only drawback is that some very small, labelled, oligo-nucleotides are produced which can increase background if not removed. However, even with 'nick-translation' the labelled DNA is almost always separated from the unincorporated nucleotides (normally by gel filtration) before the probe is used and so, with a little care at this step, the problem can be solved.

However, the real advantage in using 'oligo-labelling' comes in the insensitivity of the Klenow fragment to contaminating agarose. Because of this property, the insert does not need

to be purified from the gel fragment before labelling. All that is required is that the slice of gel (containing the insert only) be solubilised which can be achieved easily by boiling with three volumes of sterile distilled water when low gelling temperature agarose has been used to make the gel. This mixture can then be kept in the freezer until an aliquot is required for labelling, when the sample is heated to 65°C to ensure solubilisation before the aliquot is removed.

All the probes used here were prepared and labelled in this manner, by digestion to remove the insert, separation on low gelling temperature agarose gels and labelling of aliquots by 'oligo-labelling'. In all cases the labelling molecule was $\alpha^{32}\text{P}$ dATP. This high energy β emitter is most often used for restriction fragment analysis, usually because of its high energy and its short half-life (16 days - reducing disposal problems). It is incorporated very efficiently into DNA, and, when used in conjunction with intensifying screens, produces strong signals by autoradiography.

There are two main advantages of using radioactive molecules over non-radioactive labelling molecules at present, although rapid development may soon change this. Firstly the incorporation is easier to estimate (using radiation monitors) and the separation of labelled DNA fragments from unincorporated nucleotides by passing through gel filtration columns is much easier to follow (although few Geiger tubes work for tritiated nucleotides). Secondly, and more importantly, the sensitivity of detection can only be

matched by non-radioactive labels when amplification steps are introduced (eg. antibody sandwiches) which increase background or by using enzymic detection that irreversibly stains the filters (Buganan et al 1988). The fact that filters can be probed many times with radioactive labels greatly reduces the time and effort required for this work. Until this aspect can be achieved by use of non-radioactive labels, high energy radionucleides will continue to be the first choice for such work.

4.5 Hybridisation

This is the process whereby the labelled probe sequence is allowed to bind to homologous sequences on the filter, subsequently revealing their position and size. It is achieved by incubating the filter in a solution containing the denatured probe. High sodium ion concentrations in the solution stabilise the DNA-DNA duplexes at the high temperatures needed to achieve rapid re-annealing of the DNA. The melting temperature of the duplexes is affected by the G-C content of the DNA and by the length of the probe (these affect the number of hydrogen bonds) as well as the sodium ion concentration, but these are usually kept constant in most cases (for more details see Hames and Higgins 1985).

Hybridisation is usually carried out at temperatures 15 - 20 °C below the melting temperature, which, for the normal sodium concentrations of 0.5 - 0.8 M, is about 60 - 65 °C. Washes are then carried out more stringently to eliminate non-specific binding of DNA fragments - melting temperature

is reduced by 1.4 °C for every 1 % mismatching of DNA sequences. Thus to allow hybridisation of sequences with 95 % homology the washes are performed at 7 °C below the melting temperature.

Calculations such as these are rarely made, and rather the conditions can easily be determined empirically. The conditions are determined by washing in gradually increasing stringencies (lower sodium ions and higher temperatures) with autoradiography between each wash. In this way a balance can be found between background, signal intensity (signal will decrease with washing even when homology is high), and allowed homology. This method can be used to produce estimates of homology between non-homologous probe and target DNA and gives the best practical conditions for washing of filters.

The duration of the hybridisation is another parameter which, while calculation is possible, is usually kept fairly constant. Factors which affect this are the probe concentration and length, as well as temperature. Temperature has been dealt with above and is usually quite fixed because of stringency constraints. Probe length is usually quite long to increase stability of duplexes. Although the rate of annealing is faster if shorter probes are used, the reduction in stability is not usually allowed and so this parameter is also fairly constant. The amount of labelled probe is usually kept as low as possible, both to reduce the unpleasantness of working with large amounts of high energy radionucleides and to reduce costs, so the only parameter

that can reasonably be altered here is hybridisation volume - to have the probe as concentrated as possible.

To keep this volume as low as possible, yet also to keep the filter completely covered to prevent it from drying out, hybridisations are routinely carried out in plastic bags, sealed with all the air removed. The sealing and re-opening of plastic bags containing highly radioactive solutions is not particularly pleasant and so systems have been developed to reduce these manipulations as much as possible. Towards the start of this study, a system was purchased from Hybaid Ltd, which, essentially, consists of PVC bags with integral ports through which solutions can be introduced and air removed. The filters are sealed in the bag before any solutions are added and so the manipulations can be carried out with much reduced risk of contamination. Gauze meshes can be used in contact with the filters, to both aid removal of air from the bag and ensure probe solution contact is even over the filter especially when more than one filter is present in the same bag. A slight drawback with these gauzes is that they increase hybridisation volume and so if sensitivity is critical (requiring high probe concentration and long incubation to increase specific annealing of probe and target) they are often best left out. The first washes can also be carried out in the bags, which removes most of the unbound probe and also makes removal of the filters from the bag less unpleasant.

The Hybaid system works very well and has been used routinely in this study to analyse probes and check whether

variation is present in populations of regenerant plants (Figure 4.7). However, for the analysis of the large number of plants produced in the main study, a set of twenty four filters was produced, each one requiring hybridisation to the four probes. It is not possible to handle more than four filters at one time with the bag system, and so another approach was used to handle more filters. This was basically a step back to the early hybridisation protocols which were carried out in plastic boxes with sealing lids (sandwich boxes). In the past this approach required the use of large hybridisation volumes for one or two filters, but it was found that little or no extra solution was required to hybridise twenty four filters at one time, especially if the sandwich box was only just large enough to contain them. By simply adding the filters one at a time to a box that already contained the probe solution, even coverage of the filters was ensured.

The hybridisation volume used - 100 ml - was nevertheless still much greater than that used in the bags (20 - 30 ml). To keep sensitivity high in this case three things were done; i) the amount of probe was doubled (together with the amount of radioactivity to keep specific activity constant), ii) filters were hybridised (incubated in the probe solution) for twice the time, and iii) a different hybridisation buffer was used. Up till this point, the buffer used had been that recommended by the manufacturers for the membrane - although no different from the standard buffers originally used for nitrocellulose filters (Maniatis et al 1982). This buffer contained Denhardt's solution (high

molecular weight polymers) and denatured non-homologous DNA (usually salmon or herring sperm) as blocking agents to reduce the background, in addition to sodium ions, phosphate buffer, EDTA and SDS. The use of a buffer simply containing sodium phosphate, EDTA and high SDS (7 % rather than the usual 0.2 %) was suggested by Church and Gilbert (1984) to provide much greater sensitivity - without significant increase of background signals. Using this buffer (or rather a slight modification with 1 % BSA which was suggested to remove the slight increase in background observed) in the sandwich box with twenty-four filters and the conditions noted above, good signal was detected on the filters - even with the lowest copy number probes used. (See Chapter 5)

Hybridisation was carried out in shaking water baths to ensure that any air bubbles did not remain in one place on the filter and so prevent even solution contact with the filters. After initial washes (carried out in the bag where possible) at room temperature, high stringency washes were then performed in large volumes of buffer, in sandwich boxes (larger than for hybridisation to allow free movement of filters over each other) in the shaking water bath at the appropriate temperature. Washing was checked with a Geiger tube and considered complete when the edges of the filters (which should have no DNA and hence no probe bound to them) were essentially unlabelled. This usually entailed 2 x 30 minute washes in the final stringency buffer.

If filters are to be rehybridised then they must not be allowed to dry after hybridisation. The easiest way to ensure

this was found to be wrapping the filters in cling film before autoradiography (which also had the added advantage of not contaminating the cassettes and intensifying screens) and storing in a freezer at -20 °C after autoradiography until required again, when they would be removed from the cling film and stripped of the old probe before re-hybridisation.

Autoradiography was carried out in cassettes with X-ray sensitive film and two intensifying screens, maintained at -80 °C - to increase the signal from the high energy β particles emitted by the ^{32}P (Maniatis et al 1982). The cold temperature slows the particles down slightly, while the intensifying screens emit many slower particles when struck by the β from the ^{32}P . The loss of resolution that is caused by this amplification is more than made up for by the increased sensitivity. The time of exposure clearly depends on the amount of bound probe on the filter and can be estimated from Geiger tube readings. As filters can be exposed a number of times, the first exposure is usually a minimum time to see any signal - with longer exposures later if required. However, one factor to be taken into account is the short half life of ^{32}P - only 16 days - and so very long exposures (more than two weeks) are of little use.

Filters can be stripped of probe before re-hybridisation by washing at high temperatures (80 - 90 °C) in very low salt buffers (often just Tris and EDTA). In developing these techniques, filters have been hybridised as many as 10 times with good results - although the last hybridisations were

with repetitive probes and so did not require the greatest sensitivity. It is claimed by many manufacturers of membranes that sensitivity is not affected after 10 - 15 rounds of hybridisation but this has not been rigorously tested in this study.

4.6 Discussion

In this chapter the principles of restriction fragment analysis have been described, and methods identified or developed suitable for the application of such analysis to the study of stability/variability in *in vitro* manipulations. Some evidence has been given for the improvement achieved by the adoption of newer or different protocols, but most of the advantages have been in speed and ease of use - which are more difficult to quantify and show evidence of. It must be stressed that the methods employed are by no means definitive or final. In different laboratory conditions different methods may prove better - even for analysis of the same question, while study of restriction fragments for another purpose will require, perhaps, a different approach entirely. Also, developments in this area are very rapid and, driven by large interest (and investment) in human gene mapping, likely to change greatly in the next few years.

The next chapter describes the results of restriction fragment analysis, using the final methods detailed above, and applied to the plants produced as outlined in Chapter 3. The developmental work in this chapter was carried out on

some of the plants produced for this analysis, but mainly on other plants maintained at Rothamsted for other projects (eg. Foulger 1987, Fish 1988) and as such are not described in the results in the next chapter.

Chapter 5. Restriction fragment analysis to assess stability of potato *in vitro*

5.1 Introduction

As described in the previous chapter, restriction fragment analysis provides a powerful method for the genetic analysis of large numbers of plants. This chapter contains the results of such an analysis of potatoes regenerated from different *in vitro* regimes to assess the effect on genetic stability of these *in vitro* regimes.

Detailed experimental protocols are given in Chapter 2, and the particular methods for production of plants are described in Chapter 3, and those for restriction fragment analysis in Chapter 4. In this chapter the probes used are listed and the results are collated as the number of altered restriction fragment patterns within each group of plants. The complete results (normal or altered banding pattern for each plant with each probe/enzyme combination) is provided in Appendix II.

5.2 Probes and procedures used

The four probes used are given in Table 5.1, with their size, type, approximate copy number and other data. Only inserts, separated from the vector on low-gelling-temperature agarose gels, were labelled and used for hybridisation. Approximately 100 ng of SS12, SS15 and pGM01 were labelled with 100 μ Ci α^{32} PdATP, while 100 ng of 4324

Name of probe	Type	Vector	Insert size/ type	Approximate copy no.	Gene
SS12 ^a	cDNA	pUC9	≈ 1000 bp PstI frag.	Low/single	Random
SS15 ^a	cDNA	pUC9	≈ 800 bp PstI frag.	Low/single	Random
pGM01 ^b	cDNA	pUC9	≈ 1.4 kbp PstI frag.	30 - 50	Patatin
4324 ^c	genomic coding gene	pJL34	≈ 2.65 kbp EcoRI frag.	Repetitive	Ribosomal

Table 5.1 List of probes used

Source: ^a - isolated from a cDNA library of potato prepared by M Burrell, RES, ^b - from W Park, Texas, ^c - from H Uhrig, MPI Cologne.

was labelled with 50 μ Ci α^{32} PdATP together with an equivalent amount of 'cold' dATP. Hybridisations were carried out with the complete set of 24 filters (ABI, ABII, CDI, CDII, EHI, EHII, OST, CST, all with EcoRI and HindIII, V()I and V()II with BamHI, EcoRI, HindIII and XbaI - see tables in Chapter 3) in a sandwich box with 100 ml of Church hybridisation buffer as described (Chapter 2). Washing was usually in 0.5xSSC, 0.2% SDS at 65°C, but sometimes stringency was increased to 0.2xSSC at 65°C where required. Exposure times for autoradiography were varied as required, and ranged from 4 hours to 5 days.

5.3 Results

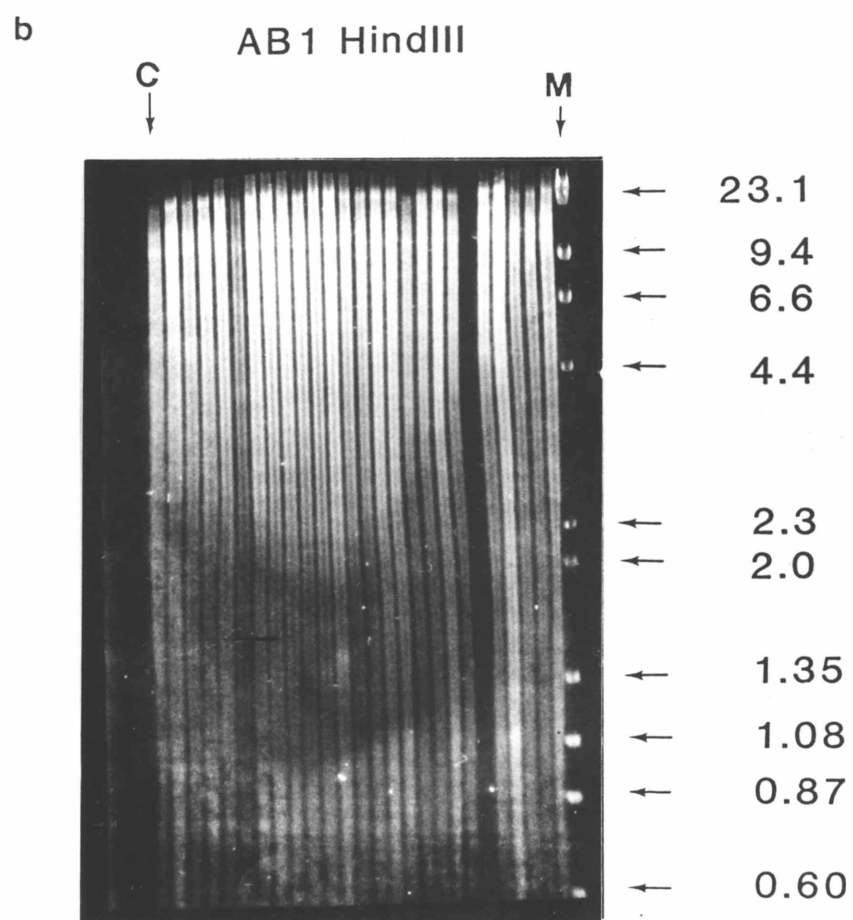
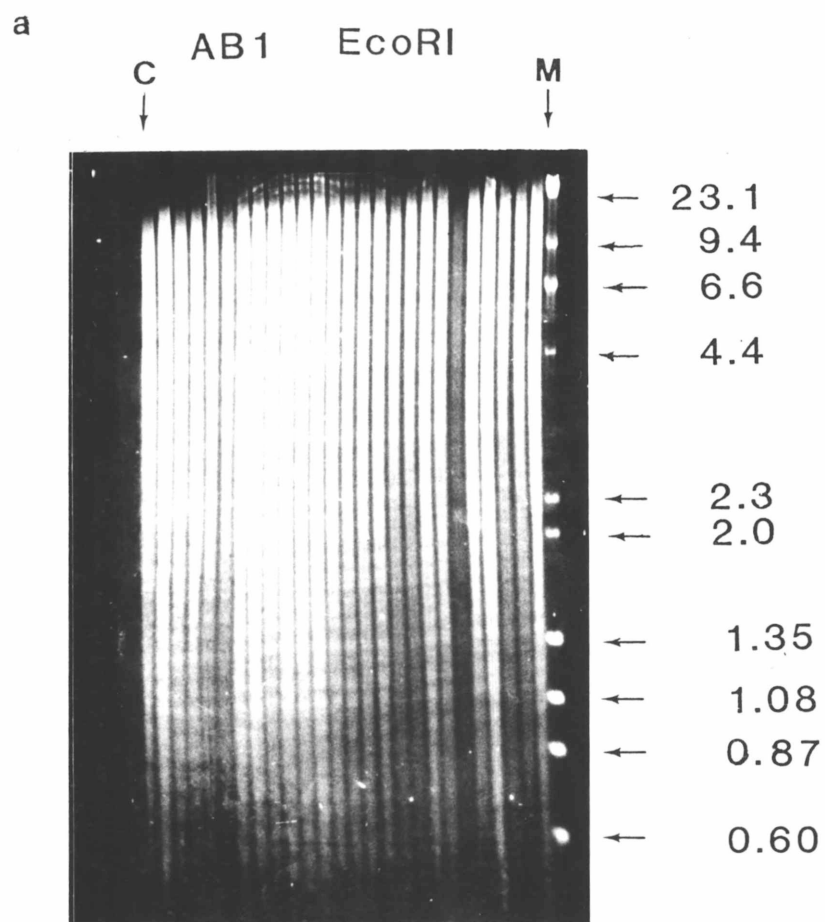
Figure 5.1 shows gels of the same DNA samples cut with two different restriction enzymes (EcoRI and HindIII) with DNA molecular size markers indicated. These gels were used to produce filters AB I EcoRI and AB I HindIII as labelled.

Figure 5.2 shows autoradiographs of AB I EcoRI probed with the four probes, and Figure 5.3 shows autoradiographs of AB I HindIII probed with the four probes. The reduction in size is by a factor of 2 in all three figures and so they are directly comparable.

These figures show the results in their basic form, i.e. autoradiographs of the filters probed with the different probes. They were then analysed by comparing the DNA restriction fragment patterns of the experimental plants with those of the controls (present on all filters, marked c on figures). Patterns similar to the controls were classed as normal, and altered patterns were classed as variants. Alterations took the form of missing bands, new bands or markedly different relative band intensities within a single track (because DNA loadings were not always identical between tracks).

Figure 5.1 (following page) Gels used for filters AB1 EcoRI and AB1 HindIII

a (top) is produced by digestion of the DNA with EcoRI, b (bottom) is produced by digestion with HindIII. C are control DNA lanes, M are molecular size marker lanes. Note the difference in intensity of some lanes, because of differences in amount of DNA loaded. Compare these with the autoradiographs following (Figs. 5.2 and 5.3) produced from these gels. See text for more details.



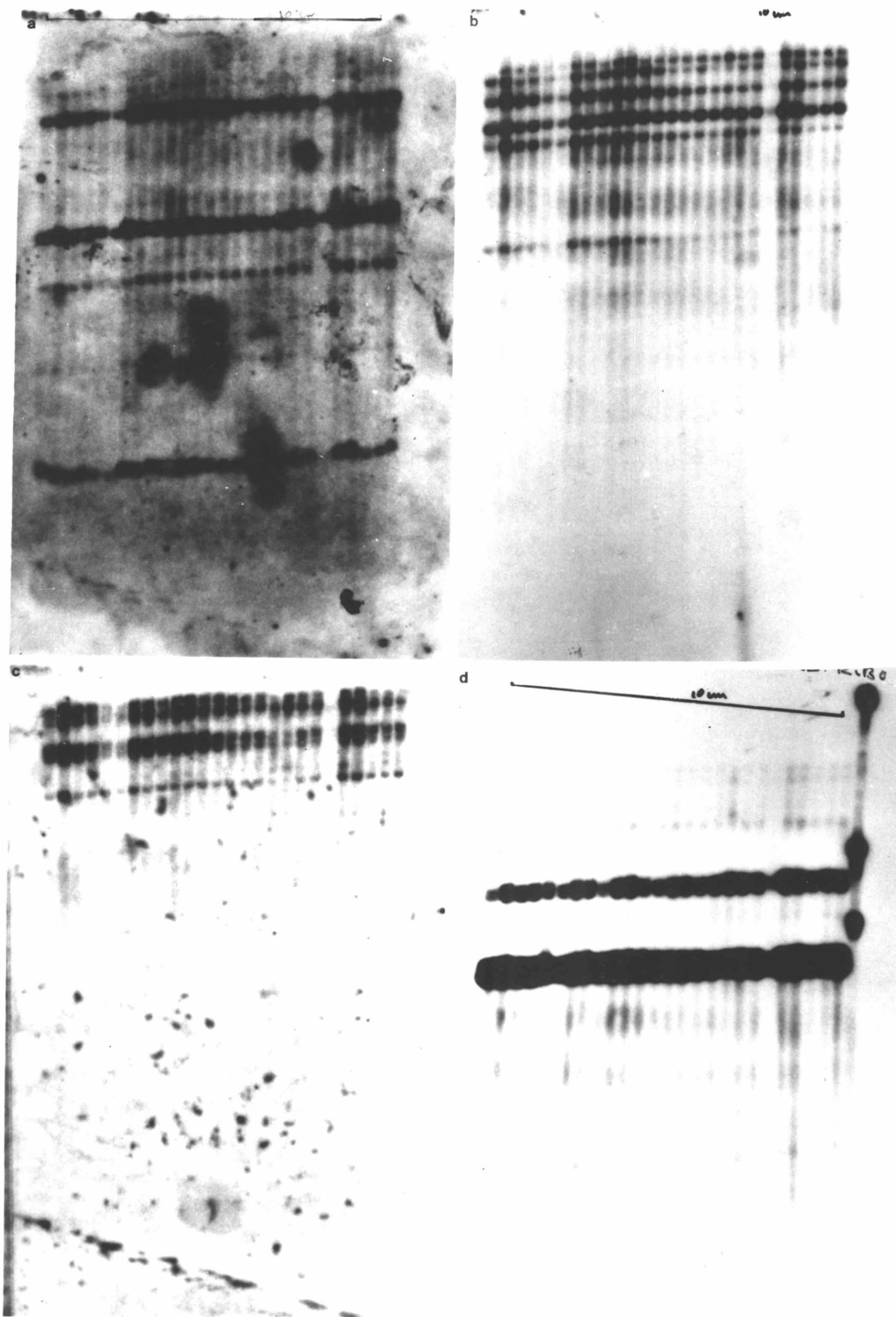


Figure 5.2 Filter AB1 EcoRI probed with all four probes
 Autoradiograms of the filter probed with: a - SS12, b - SS15, c - pGM01, d - 4324. Whole autoradiograms are shown for comparison with Fig. 5.1a (same size reduction)

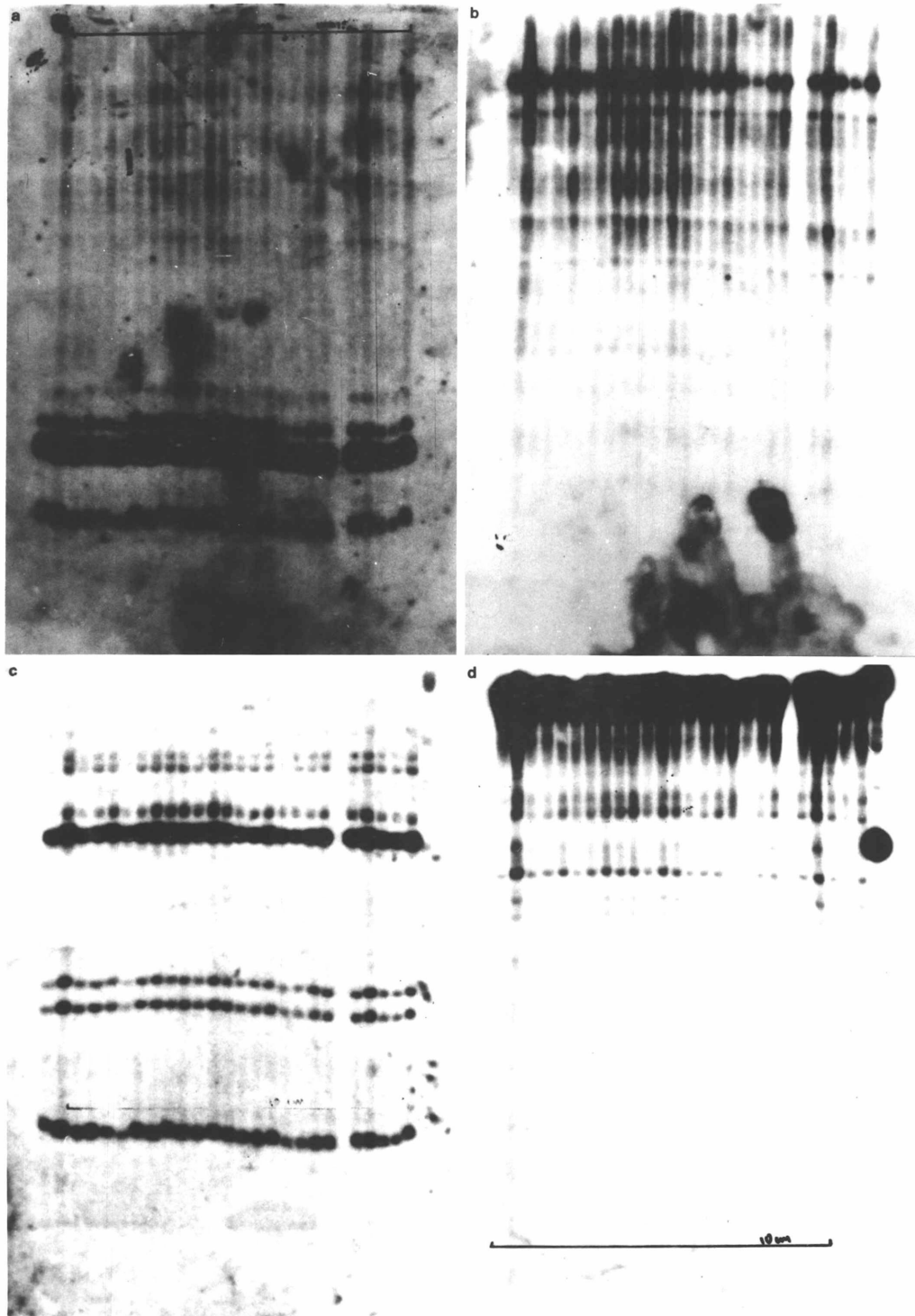


Figure 5.3 Filter AB1 HindIII probed with all four probes
 Autoradiograms of the filter probed with: a - SS12, b - SS15, c - pGM01, d - 4324. Whole autoradiograms are shown for comparison with Fig. 5.1a (same size reduction)

It can also be seen from these figures that it was not possible to score every track, as a result either of low amounts of DNA (as shown by Figure 5.1) or of poor hybridisation. Because of this, not all of the possible data was collected. In an analysis on this scale, it was not feasible to repeat work for the sake of one or two indistinct samples, and since the results will be assessed statistically, it is possible to allow for missing data.

All the results - presented either as normal patterns (+) variant patterns (X) or missing data (-) - are presented in Appendix II and are summarised in Table 5.2 after collecting the data for for each individual group of plants. The number of probe/enzyme combinations refers to the number of different probes used, multiplied by the different enzymes used to digest the DNA. The stored and micropropagated DNA samples were cut with EcoRI and HindIII (8 probe/enzyme combinations), while the DNA of the callus derived regenerants were cut with BamHI and XbaI as well as the previous two restriction enzymes (16 probe/enzyme combinations). Multiplied by the number of plants analysed in each group, this gives the total number of possible data points. But, since there were some not scored, the actual number of points obtained is given in the next column. Overall, the number of data points actually scored was over 79% of the total possible.

Table 5.2 also indicates the number of variant patterns found for each set of plants. No variants were found in any of the meristem-based classes (micropropagation, cold- and

<u>PLANT SET</u>	<u>No. OF PLANTS</u>	<u>PROBE/ENZYME COMBINATIONS</u>	<u>PLANTS X P/E COMB.</u> <u>TOTAL</u>	<u>ACTUAL</u>	<u>No. OF VARIANTS</u>
MICROPROPAGATED	128	8	1024	866	0
COLD-STORED	18	8	144	141	0
OSMO-STORED	21	8	168	100	0
REGENERANTS	46	16	736	530	9
TOTALS	<u>213</u>	-	<u>2072</u>	<u>1637</u>	<u>9</u>

Table 5.2 Summary of restriction fragment data

osmo-stored), whereas 9 variants were found in the regenerant class derived from callus. Taken together, this regenerant class significantly differs from the other three classes at greater than 5% confidence limits (K Moore, Statistics Department, RES. Data not shown). Only 2 variant patterns were found for the same plant using the same probe with different restriction enzymes (Appendix II, filter V()II). This implies that the same mutation is being picked up on both occasions. Thus there were 8 separate mutations found. On 2 occasions 2 mutations were found within the same plant (although with different probes and so separate mutations) giving 6 identified variant plants (see Appendix II, filters V()I and V()II).

Some individual variants have already been shown in Chapter 4 (Figure 4.7), identified during the development of the restriction fragment analysis. These were confirmed during the complete analysis performed as above, and 2 of the other variants identified are shown in Figures 5.4 and 5.5. Figure 5.4 shows 2 new bands in one lane (arrowed), suggesting a new restriction enzyme recognition site producing 2 fragments from only one initially. This is confused slightly by the fact that no other band appears to be missing or reduced in copy number. However, this probe, pGM01, has approximately 30 - 50 copies in Desiree (Twell & Ooms 1988) and so loss of one copy from one of the heavier bands (≈ 10 copies) would be hard to identify.

In Figure 5.5, the variation in relative intensity of 2 bands is shown (arrowed). It is difficult to see this reduction in intensity in photographs of the autoradiograph and so, in order to show this more clearly, densitometry was used to analyse the autoradiograph itself. Using a scanning laser densitometer (Ultrosan XL, LKB) measurements of the density of the bands in three tracks were taken, the variant track and a track on either side. The data were transferred to a VAX mainframe computer (RES Computing Unit) and plotted using 'gelplot' (A M Ball, RES Computing Unit) to produce Figure 5.6. The altered bands (now shown as peaks on the curve) can clearly be seen.

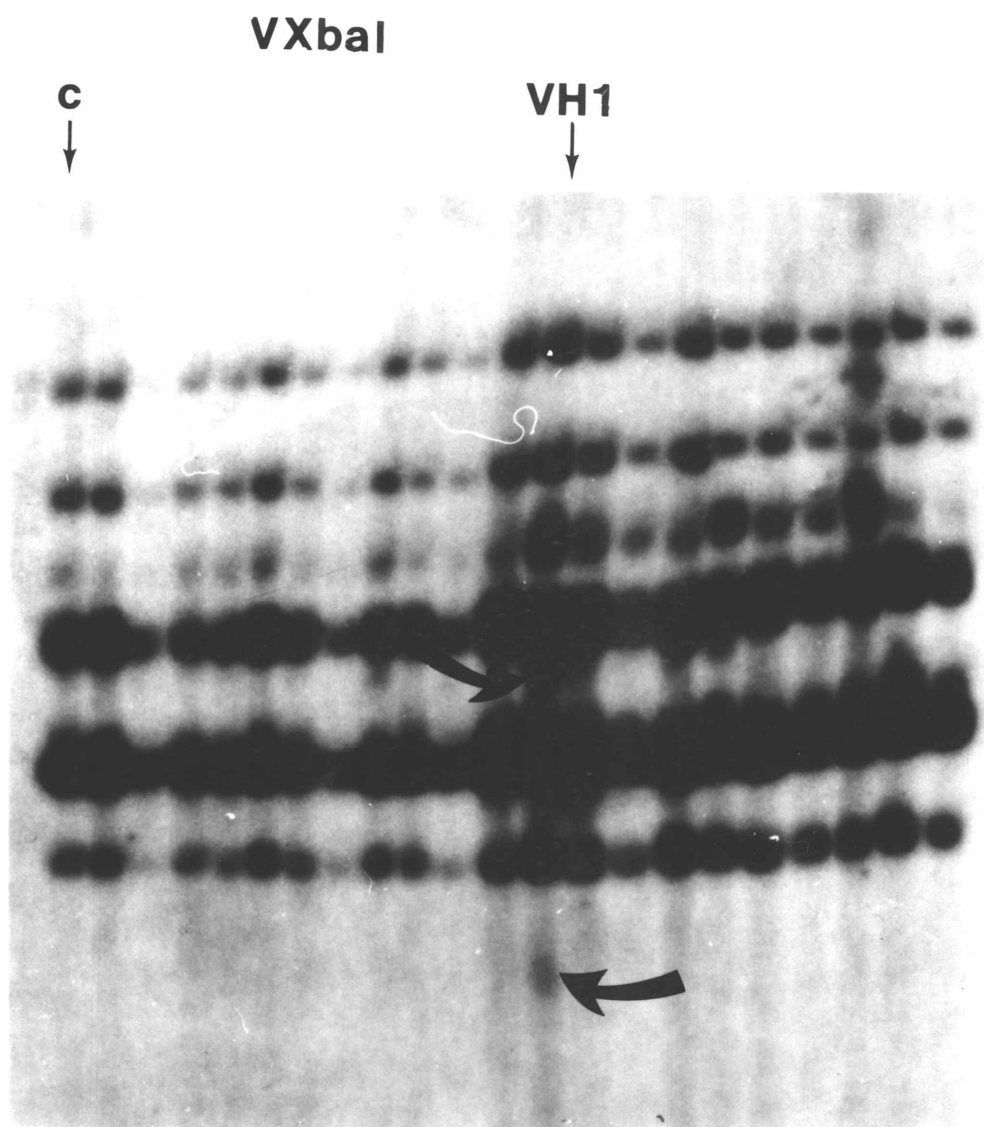


Figure 5.4 Filter VXbaI probed with pGM01
 The extra bands in lane VH1 are arrowed. c is the control lane.
 See text for further details.

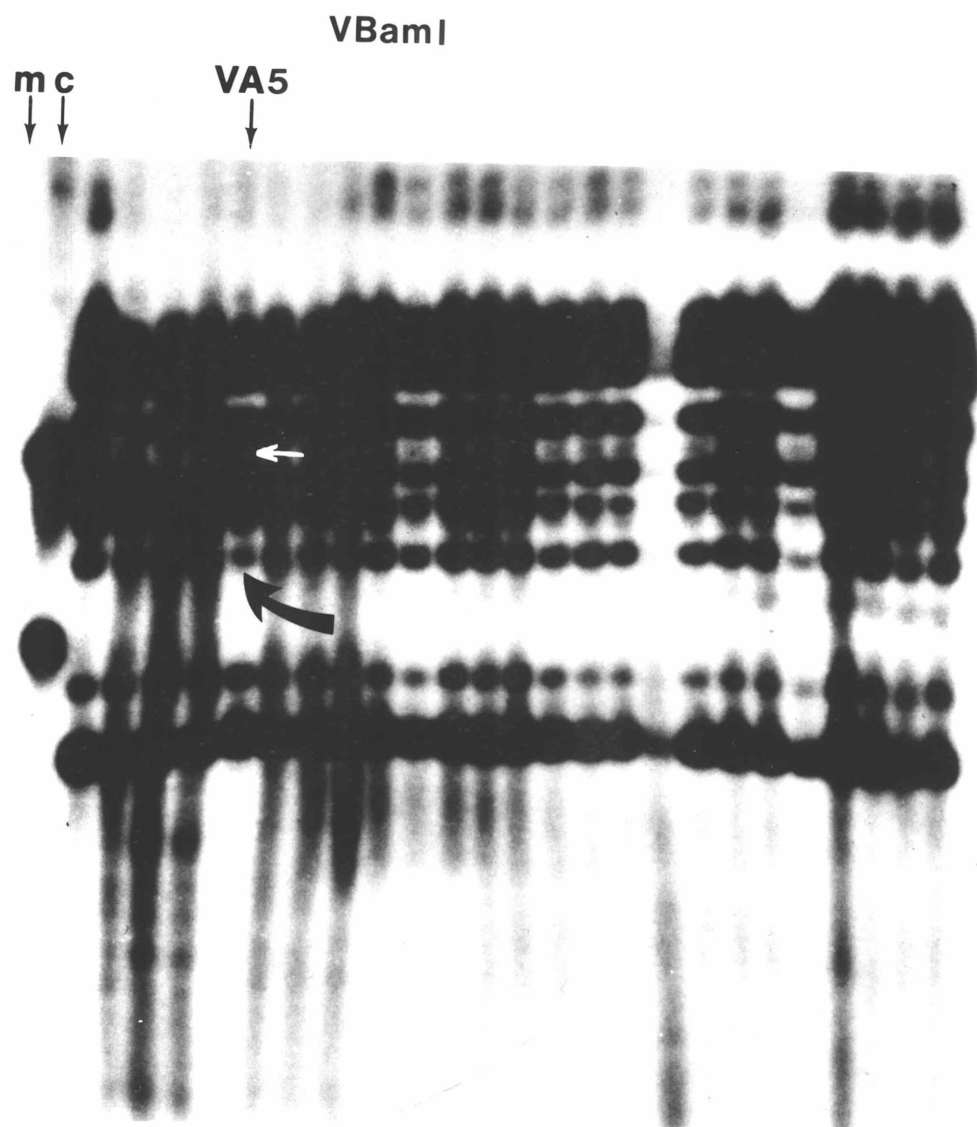


Figure 5.5 Filter VBamI probed with 4324

In lane VA5, one reduced intensity band is arrowed in black, and another increased intensity band is arrowed in white. This is not very clear from the photograph, and so densitometry was carried out. See text and Fig. 5.6 for further details.

VBamI, 4324

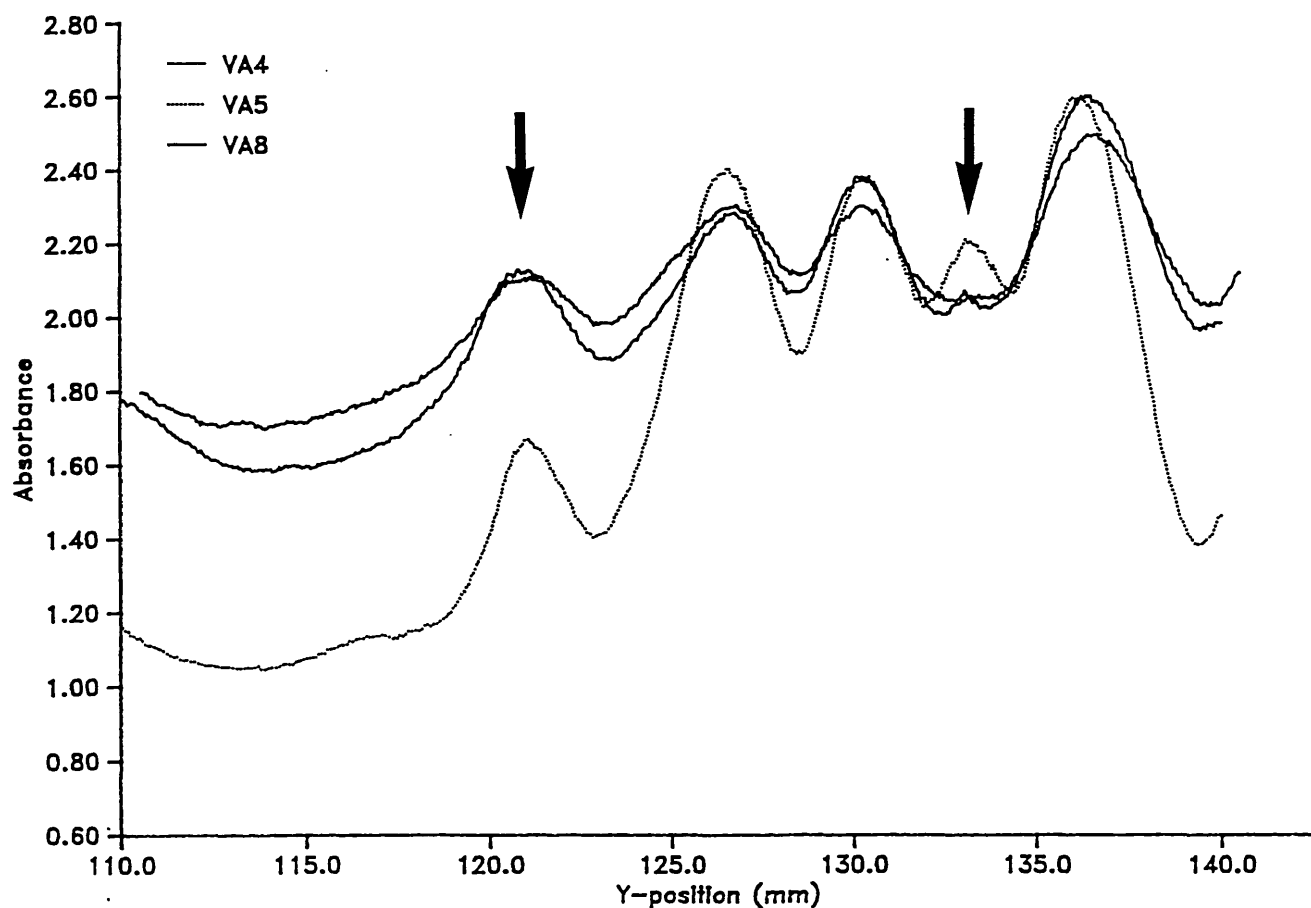


Figure 5.6 Scanning densitometer trace of VBamI

The three lines correspond to the altered lane in Fig. 5.5, and the lanes either side. Band of reduced intensity is arrowed, left, and the band of increased intensity is arrowed, right. More background in lanes VA4 and VA8 has increased the baseline, but the altered peak heights are clearly shown.

5.4 Discussion

Based on these data of restriction fragment analysis, it can be said that regeneration from intact meristems is more stable than regeneration via a callus phase. However, the data are not extensive enough to give an idea of how much more stable, or whether the various different storage regimes are more or less stable than straightforward

micropropagation.

The number of variants found was too small to draw conclusions on whether any particular combination of restriction enzymes or probes are more useful in finding variation. Over half the variant patterns were found with probe 4324, but this is perhaps not surprising, since not only is this the most repetitive of the probes used (and hence covers the largest proportion of the genome), but it also codes for ribosomal genes, which have been shown to be quite variable by many workers (eg. Rogers and Bendich 1987 a & b). The altered gene copy numbers illustrated by this type of probe are more difficult to identify and so further analysis of the autoradiographs (by densitometry rather than just by eye) is recommended. It may be possible to automate this to the extent that analysis of peaks is done by computer, programmed to detect variation when relative peak heights fall outside pre-set limits.

With lower copy number probes, variation is more easily detectable as a result of gain or loss of bands, but such changes are found less frequently. It is preferable to study such clear cut changes in banding patterns, but more plants need to be screened with more probe/enzyme combinations. When low copy number probes are used, it is possible that loss of a single band is the result of loss of a single chromosome. Such occurrences are common in callus-derived regenerants. Chromosome analysis in potato is quite difficult, because the chromosomes are small. Changes in ploidy level (from tetraploid to octaploid for example) are

not so difficult to record, but loss or gain of a single chromosome requires more skill to identify with certainty. The chromosome numbers of 30 of the callus-derived regenerants were counted by Dr A Karp (RES), and all were found to be euploid ($2n = 4x = 48$). Previous work at RES (Wheeler et al 1985) suggests that more than 90% of potato plants regenerated from callus by the procedure used would be euploid; with the remainder mostly doubled up to the octaploid level. Such changes can produce gross phenotypic aberrations, but none were seen in the regenerant population, possibly because such plants grow poorly in culture and may have been discarded. This does not, however, eliminate the possibility that single chromosome loss had occurred in a plant such as VD3 where loss of a single EcoRI band was seen with probe SS15.

Although this work has taken about three years to complete, the restriction fragment analysis has been developed to the point where further analysis - including DNA extraction - could be carried out in - perhaps - 6 months. Thus, the approach described now represents a rapid technique for analysing large numbers of plants, with the possibility that the analysis could be taken much further by repeated hybridisations on more filters. These could be readily produced from the already extracted (and in some cases) restricted DNA.

Another type of DNA alteration which has been implicated in somaclonal variation is DNA methylation. To assess the methylation state of DNA, pairs of restriction enzymes are

used which, although they recognise the same base sequence, are affected differently by methylation of adenosine or cytosine residues within the recognition site. This can be analysed at the whole genomic level, by comparing the appearance of digested DNA on gels (i.e. the average size of the restricted fragments) (Brown and Lörz 1985), or specific sites can be analysed by blotting and hybridising with probes (Quemada et al 1987).

The methylation state of the DNA has not been analysed in this thesis, because the techniques used to assess this are somewhat different from those used to assess restriction fragment lengths. The difference mainly involves the extraction of DNA, since a higher purity is required for the assessment of methylation state, in order that inhibition of the restriction enzyme by impurities in the DNA sample can be ruled out.

It is also hard to reconcile the stable, and often quite small, phenotypic changes associated with somaclonal variation, with the gross changes in methylation of DNA observed in callus and regenerated plants (Brown & Lörz 1985). Although methylation enzymes are believed to work in a semi-conservative manner (methylating bases of a newly synthesised strand of DNA where methylated bases are found on the template strand), methylation could conceivably be considered as a transient alteration in the DNA. Thus methylation may be involved in some of the unstable phenotypic effects seen in some regenerated plants, sometimes referred to as epigenetic changes. For these reasons outlined above, it

was decided that assessment of DNA methylation would not be part of this study.

Chapter 6. Assessment of stability by electrophoresis of soluble tuber proteins.

6.1 Introduction

Genotype identification of crop plants is usually done on the basis of morphology of the whole plant in the field. A disadvantage of this approach for evaluation of harvested crops is that another field generation is required before evaluation. Methods using biochemical markers have therefore been developed to speed up this process. In cereals, seed proteins are routinely analysed by gel electrophoresis, especially since the quality of the seed for different uses can also be established by analysis of the storage proteins in the endosperm. For the potato crop, its quality does not depend on the proteins in the tubers, but a similar analysis of proteins in the mature tuber is a useful approach for the identification of varieties based on the tuber alone.

A routine method for extraction of soluble tuber proteins and analysis by electrophoresis on non-denaturing gels has been developed to the point where an index has been produced of all classified European potato varieties, that contains distinctive protein spectra (Stegemann & Loeschke 1976, Stegemann & Schnick 1982, 1985). Variation in the protein spectra of tubers is thus of great importance as this could alter the classification of the variety. Smith (1986) identified somaclonal variants in protoplast-derived populations of potatoes using such analysis, and so the approach appears to be useful for the study of stability of plants from in

vitro culture.

6.2 Methods

To analyse tuber proteins, the protocols of Stegemann & Schnick (1986) have been followed with slight alteration (see chapter 2). A press was not available, so sap was extracted by homogenisation and then squeezing the tissue through two layers of muslin, before addition of sulphite solution. All electrophoresis solutions were as described by Stegemann and Schnick (1986), but the electrophoresis apparatus used was a Protean II cell (Biorad). This apparatus has a central core with circulating coolant and gels were run at a constant 4°C and with a constant 30 mA per gel from a Chandos current controlled power pack.

These slight differences in procedures had the result that the protein spectra produced, after staining for total proteins with Coomassie blue, were not directly comparable with those of Stegemann, and so his classification system could not be used. For the purposes of this investigation into stability of plants after *in vitro* culture, classification was made on the presence of nine particular bands (fig 6.1). These were chosen because they were well separated in the middle portion of the gel as run under the particular conditions used.

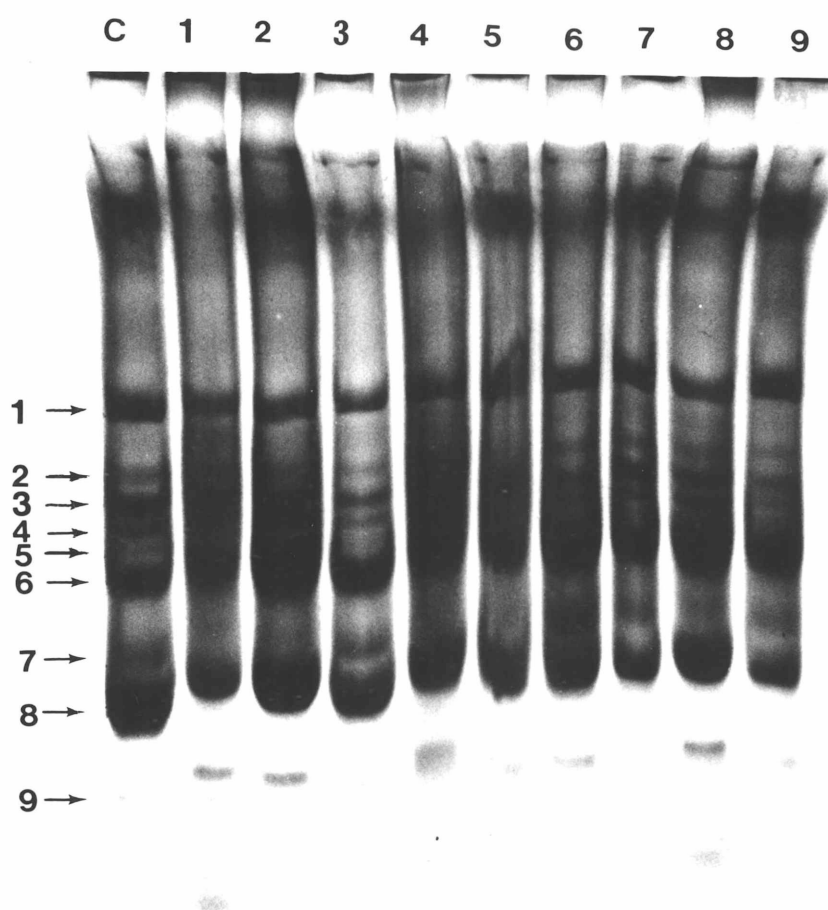


Figure 6.1 Soluble tuber protein gel

The 9 bands used for analysis are marked down the left hand side. c is the control lane. Note alteration in bands (eg. band 5 missing in lane 3).

6.2.1 Plant materials used

The source of the tubers used for the analysis is given in chapter 3. Tubers from the first pot-grown generation (transplanted from culture) of the leaf-disc regenerants were initially used, but the protein spectra produced from these tubers were variable - even in tubers from the same

pot! This was as a result of physiological differences, perhaps caused by carry-over effects from tissue culture. A second (pot-grown) generation was therefore grown for all the callus derived plants, and for as many of the other plants that could be managed. Tubers from these plants were then used for protein analysis. Desborough (1985) reported no change in tuber protein pattern once tubers had reached a size of one gramme or more, but this was using an SDS buffer system which only shows individual subunits, not the native proteins in their aggregated form. As this aggregated form is likely to be more related to maturity (and therefore size) tubers over 5 g were normally taken for analysis, although this was not always possible.

Native proteins from *in vitro* tubers were also extracted and investigated, but again the protein patterns were found to be very variable (probably due to the same problems as mentioned above). Again, a pot-grown generation was used to provide more comparable material. However, unpredictable dormancy of some of the *in vitro* tubers was a problem, and as a result the cold-stored plants were not subjected to the protein analysis. The exact tissue source for each of the clones analysed is given in Chapter 3 in the tables at the end of that chapter.

6.3 Results

A complete list of the results obtained from the analysis of the non-denaturing soluble tuber proteins obtained by electrophoresis is given in Appendix III. This is grouped

by class of plants produced and gives the weight of tuber used for analysis and records the results as presence (+) or absence (-) each of the nine bands specified (Figure 6.1). In some cases extra bands were seen within a region of the gel normally occupied by only one band, this is represented by a 2 in the Tables in Appendix III.

<u>PLANT SET</u>	No. PLANTS <u>TESTED</u>	No. PLANTS <u>VARIANT</u>	<u>No. VARIANT IN EACH BAND</u>								
			BAND NUMBER								
			1	2	3	4	5	6	7	8	9
MICROPROPAGATED	129	98	-	2	-	6	50	3	68	1	1
STORED	16	12	-	-	-	1	11	-	3	-	-
REGENERANTS	44	33	5	1	1	3	19	3	17	-	-
TOTALS	189	143	5	3	1	10	80	6	88	1	1

Table 6.1 Summary of protein electrophoresis data

The data is summarised in Table 6.1, from which it can be seen that considerable variation in protein spectra were found for all sets of plants analysed. Approximately 75% of the plants in each group exhibited a distinctly different band pattern having either bands missing or sometimes extra bands present. This situation was also found for micropropagated plants (Figure 6.1) in which lanes 1 and 2

are missing band 7 and lane 3 is missing band 5. This proportion of variant plants is much higher than expected and shows no difference between the groups. The only exception to this was that the callus-derived regenerants showed variation in nearly all bands whereas the variation was mainly restricted to two particular bands in the micropropagated plants.

6.4 Discussion

The high proportion of plants that exhibited variant protein spectra, and the absence of significant difference in this proportion between the callus-derived regenerants and the meristem derived plants suggests that this is not a reflection of the genetic constitution of the plants. It is more probable that this variation in banding patterns is a reflection of physiological differences as a result of environmental effects. These include differences in tuber size and growth conditions such as daylength, temperature and time of harvest. The latter two are probably more important since the plants were grown in a greenhouse with an automatically controlled blackout, to maintain a constant daylength.

It was noted during extraction that there was a difference in consistency of the homogenate from different tubers, probably caused by a difference in water content in the tubers. This would result in a lower or higher protein concentration in the extracted sample (if protein content was constant in the tuber) which was seen in the gels (see

Figure 6.1) as lighter or heavier bands. This does not, however, account for the complete absence of bands in some - otherwise heavily stained - samples, or for the appearance of new bands.

The much greater stability referred to by Stegemann and others, is probably related to the greater uniformity of tubers grown over many generations in the field. Similar environmental conditions and harvest times (in relation to maturity), will produce a far more consistent sample. The uniformity in pattern from even quite small tubers mentioned by Desborough (1985) is not comparable as this was based on SDS extracted proteins and not the aggregates analysed here which are likely to be more affected by maturity.

These results lead to the conclusion that, for the purposes of this study, this type of analysis is of little use unless carried out on mature, field-grown material. The use of such protein spectra in cultivar identification and evaluation of somaclonal variation shows a strong genetic component in the make-up of the protein complexes. For this reason, it should be an important method for analysis of genetic stability, however, because of strong environmental interactions care should be taken to ensure comparison of similar material.

Chapter 7. Analysis of field morphology

7.1 Introduction

As already mentioned in the general introduction, field morphology has always been and will continue to be the major functional test for genetic stability, as measured by true-to-type plants in seed tuber multiplication programmes. No other method can be used realistically to screen the numbers of plants grown each year by seed tuber producers. And, while the analysis is based purely on a limited set of morphological markers, these are the only criteria of concern to seed tuber producers because this is how their product will be judged. Thus, the morphology of plants grown in a field test was analysed, to compare this, standard, approach with the other methods of assessment.

The fact that somaclonal variation was first described in the phenotype of whole plants regenerated from culture, has meant that morphology has been a major method in assessing the incidence of such variation. Larkin and Scowcroft (1981) referred to many examples of altered morphology as the first known instances of somaclonal variation, while Shepherd et al (1980) found many variants in growth habit and tuber morphology among 10,000 somaclones of potato cultivar Russet Burbank. Most of this early work (especially that of Shepherd and co-workers) was directed towards production of new, improved, varieties. But even in academic studies on the causes and origins of somaclonal variation, morphology is often used as a method of analysis.

For example, Evans et al (1987) looked at tuber characteristics over three generations in a population of potato somaclones in order to examine which characters were altered and how stable these changes were. Gavazzi et al (1987) compared somaclonal variation and chemically induced mutagenesis in tomato on the basis of the morphology of regenerated plants and their progeny.

Thus, whole plant morphology is a valid method for the study of stability of potato after *in vitro* treatments, provided that the plants can be grown in similar environmental conditions. Because maintaining completely controlled environments over a long time period is difficult and expensive, potted plants (transplanted direct from culture) were not grown under similar conditions and so could not be used for morphology assessment. It was decided that a small field trial would be the best system for comparison of the morphology of all the plants produced because they could all be grown at the same time and so environmental interactions could be minimised for comparing the plants. Therefore, a field trial was planned in 1987 and carried out in the summer of 1988 at RES in collaboration with the small plots section of the Field Experiments department.

7.2 Materials and methods

7.2.1 Planting material

As a result of limitations of manpower available, it was decided that only single replicates of the micropropagated plants, 2 replicates of the other groups of *in vitro* treated plants and 4 replicates of the 6 original shoots (grown up as control plants) would be planted, as tubers. In order to standardise the planting material as much as possible, many of the plants were grown through a second generation in pots (from tubers produced in the first pot-grown generation). All of the callus-derived regenerants were thus treated and as many of the micropropagated plants as could be managed. The *in vitro* produced tubers were only grown for one generation in pots as these had already undergone one cycle of tuberisation. The dormancy of these tubers was very variable and difficult to break, and, as not all of the *in vitro* tubers from the cold-stored plants could be grown in pots to produce tubers in time for the field trial, *in vitro* tubers from these plants were planted direct into the field. The exact planting material is given for each plant in the Tables in Chapter 3.

7.2.2 Growth and measurement of plants

Pot-grown tubers were vernalised for at least 4 weeks at 4°C and then allowed to sprout at room temperature for a further 4 weeks before being planted by hand into ridges (plants 15

inches apart, ridges 30 inches apart) on 16 May 1988. The planting position was randomised, with each tuber being treated individually (no allowance made for replicates of the same clone). Preparation of the ground and all cultivation (except planting and harvesting) were performed by the small plots section and were standard treatments for potato. No artificial irrigation was provided.

The set of characters scored was taken from the Guide to the Identification of Potato Varieties (1975, National Institute of Agricultural Botany). In general, only discrete characters were scored as there were not enough replicates of individual clones for other quantitative characteristics to give meaningful results. The characters measured covered flower colour and morphology, compound leaf morphology and general plant colour and pigmentation. In addition morphology, number and weight of tubers were also scored. Tubers are normally grouped into size classes by sieves and riddles, but, as these were not available, weight classes were used instead with number and total weight of tubers in each weight class being determined. All the characters used are given in Table 7.1.

Plant morphology measurements were taken at time of full flowering. Because the growth of the plants and thus their maturity were so variable, the time of flowering was not scored as a characteristic. It was noted, however, so that comparisons were not made between plants with widely different flowering dates. Tubers were harvested when plants had obviously begun to senesce (over half the stems with

yellowed leaves). The harvest date was noted for comparative purposes, but was not scored as a character. Tubers were washed and dried before weighing and scoring for morphology.

I. Leaf-stem characters

1. compound leaf
2. terminal leaflet shape - length/width
3. lateral leaflet shape - length/width
4. stem wings
5. anthocyanin pigmentation
 - a-stem
 - b-leaf axil
 - c-rachides
6. foliage colour
7. foliage gloss
8. berries

II. Floral characters

1. flower colour
2. anther colour
3. anther morphology
4. protrusion of style

III. Tuber characters

1. shape
2. skin colour
3. flesh colour
4. no. and total weight
in each size class

1=	> 10 g
2=	10 - 30 g
3=	30 - 100 g
4=	100 - 200 g
5=	200 - 500 g
6=	< 500g

Table 7.1 List of morphological characters scored

7.3 Results

Complete data of all measurements taken are contained in the Tables in Appendix IV.

7.3.1 Discrete morphological variants

Figure 7.1 shows the field on the date that morphology measurements were first taken, 27 July 1988. This clearly shows the difference in the growth stage of the plants, with some barely emerging while others were in full flower. It was apparent from the early stages that many of the tubers planted were still dormant and heterogeneity was observed in the time of emergence of the shoots. It was for this reason that the time of flowering was not used as a character in assessing the plants, and comparison was only made between plants with similar maturities - estimated by the time of flowering.

Individual variant plants were identified that differed in compound leaf morphology and in flower morphology (figures 7.2 & 7.3). Figure 7.2a shows the normal leaf morphology of cv. Desiree with the laterals just overlapping the terminal leaflet. Figure 7.2b shows plant VB8 with quite a large gap between the laterals and the terminal. This marked difference was found on nearly all complete compound leaves and persisted throughout flowering. Figure 7.3a shows the normal flower type of cv. Desiree with a trace of purple on the petals and straight anthers with little protrusion of style. Figure 7.3b & c show plants VA1 and VA5 respectively which



Figure 7.1 Field trial, 27 July 1988

Note that, although many plants were in flower, many were small and a few had still not emerged. This illustrates the problems of dormancy in the planting material.

both show a similar mutation, with completely white petals and curved, slightly deformed anthers, allowing much more protrusion of the style. Both these plants produced relatively few flowers compared to the other plants that flowered at the same time.

Differences were found in many other characters, such as stem pigmentation and appearance of stem wings, but these also seemed to be affected by the maturity of the plant and so were not scored as discrete variants. In order to take this into account, however, these characters were analysed, all together, at the population level.

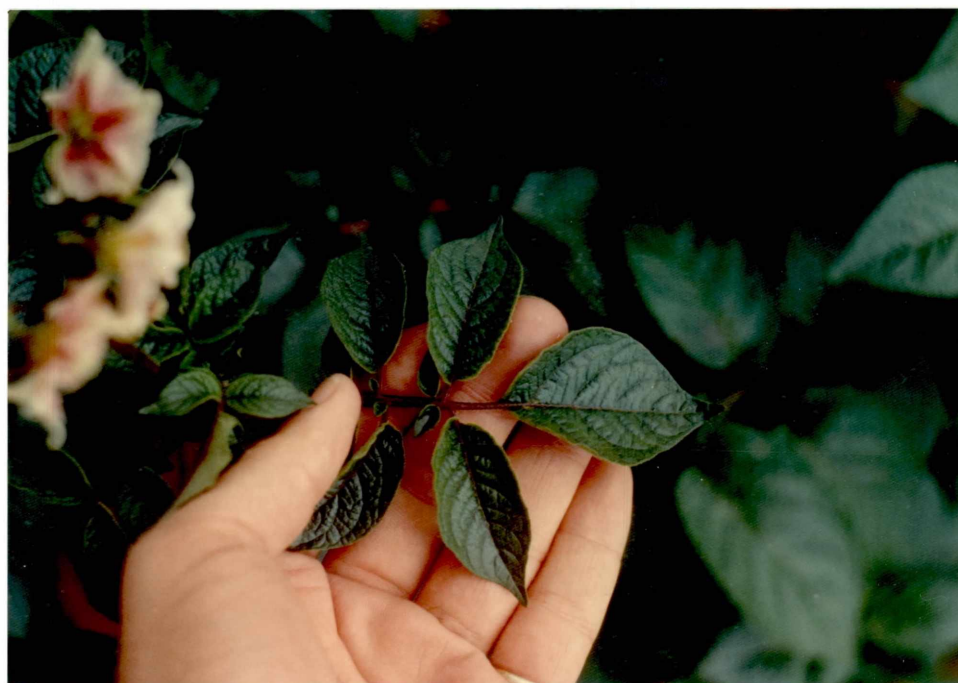


Figure 7.2 Compound leaf morphology variant
a (top) shows the normal compound leaf of cv. Desiree. b shows the variant VB8. See text for explanation.

Figure 7.3 (following page) Flower colour variants
a (top) shows the normal Desiree flower just after opening. b (middle) shows the variant VA1, with white flowers. c (bottom) shows the variant VA5 with white flowers also. The abnormal, curved, anthers can just be seen on these photographs. See text for further details.



7.3.3 Comparison of populations

As previously mentioned, the flowering date (and hence the date of scoring) was variable in this field trial, thus it was not valid to compare the entire population of plants because external conditions were not constant throughout the season. However, a significant number of plants were in full flower and were scored on the first date that measurements were taken (27 July 1988) and so can be presumed to be comparable with respect to maturity and environmental effects. These 69 plants were split quite evenly between micropropagated and callus-derived regenerants, and so provided an opportunity to compare the two treatments by assessing whole population variation, not just numbers of individual variant plants.

The populations were compared by using multivariate statistical analysis, to evaluate the variance within the two populations and then compare this with the difference between them. The analysis was carried out by K Moore and P Digby of the Statistics Department of RES using 'Genstat 5' on a Vax mainframe computer. A two-step procedure was employed, first to convert all the data into comparable forms (as characters were variable in their basis i.e. some discrete and some continuous) and then to construct a similarity matrix with the data. Population means were calculated and the distance of each individual from the mean (for its population - the two groups were treated separately) was found and grouped in a histogram (Figure 7.4). This clearly shows a difference between the two

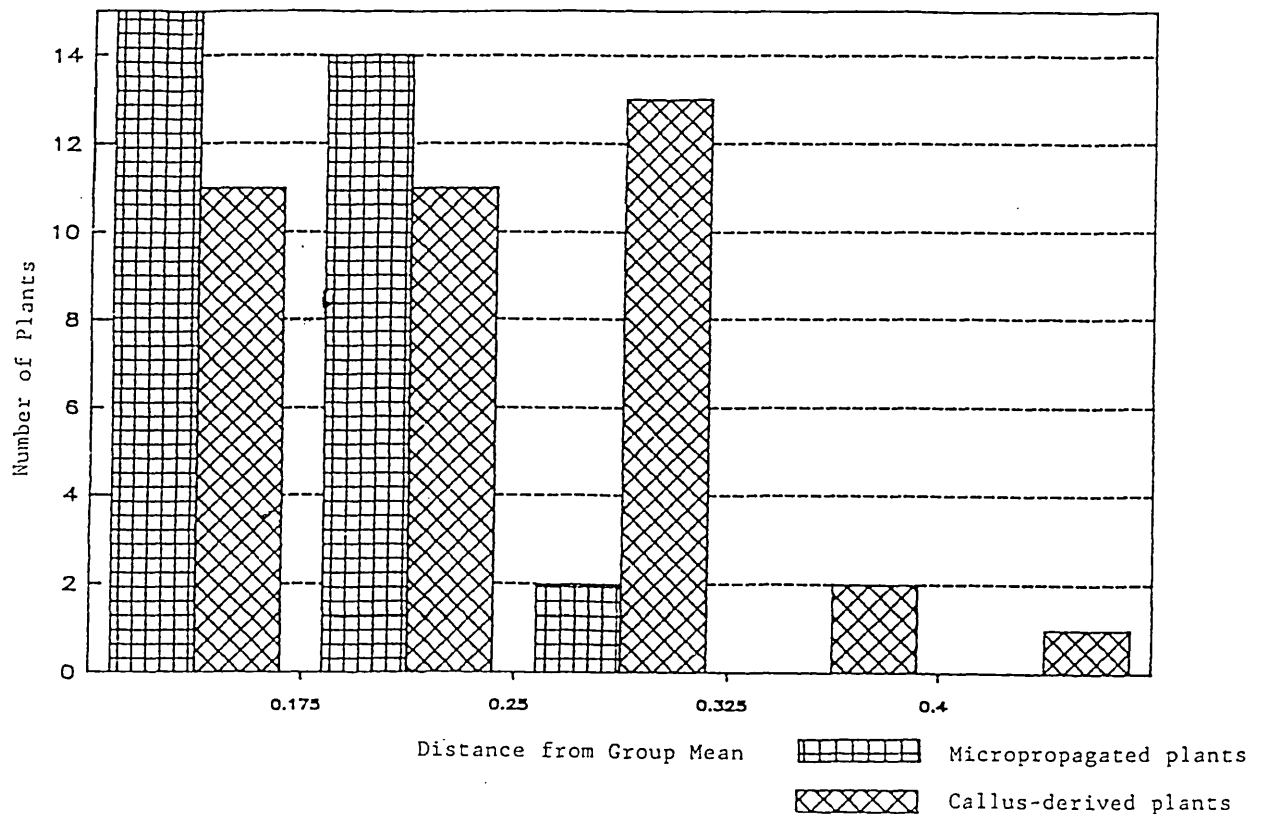


Figure 7.4 Histogram of centroid distances

Distances from group mean of plants in the two groups, collected into a histogram to show the greater spread of the callus-derived population. Note that, although nearly all of the micropropagated plants fall within 0.25 units from the mean, many plants fall outside this value in the callus-derived group.

populations which is significant at the 0.1% level - based on Tukeys Quick test. Further confirmation that the two populations were not the same was supplied by using the Smirnov test of equality of distribution function which gave a greater than 99% certainty that the populations were different.

Further details of the statistical methods used for this analysis can be found in Digby & Moore (1989) in which the above data were used to illustrate the two-step approach to comparison of similarity.

7.4 Discussion

Based on discrete characters, the only morphological variants in the field-grown plants were regenerants from leaf-disc callus, which implies that such treatment causes more instability than the other types of *in vitro* culture. However, only three such variants were identified, and this number is not enough on which to base firm conclusions. This is particularly so since many of the plants were not scored under identical conditions, because of variation in their growth. The importance of advanced planning of such field work is thus evident, so that tubers can be prepared in time for proper vernalisation and sprouting to occur thus providing consistent planting material. If this had been possible more plants could have been scored, and also more characters for each plant. Ideally for potato, in order to carry out a definitive morphological analysis, a number of years of field growth would be required.

When equivalent plants are compared, then whole population variation can also be taken into account as well as individual variant plants. Using quantitative characters - that can only be assessed for individuals in multi-replicated field trials - differences in variance can be determined between populations. As used here, the two populations were shown to be quite different, with the callus-derived population exhibiting a significantly greater amount of variation. This population approach would seem to be useful for analysing variation as it can be performed on relatively small trials with few replicates of individual

plants.

Although field assessment is thus of great use for analysis of stability (and, as mentioned earlier, will continue to be used practically in seed tuber multiplication), the fact that equivalent planting material needs to be produced, and preferably grown for a number of years, to have a valid field trial makes it slow to assess *in vitro* regimes where plants are produced all year round. The extra time needed to prepare tubers for planting (vernalisation and sprouting) after regeneration of shoots and growth of plants in pots, means that a minimum of one year is needed to produce results for field comparison of *in vitro* treatments.

Chapter 8. General discussion

8.1 Stability/variability: comparison of *in vitro* regimes

The major aim of the work in this thesis, as stated in the introduction, was to provide experimental evidence towards answering the question of whether or not potato plants regenerated from different micropropagation regimes were genetically altered. It is generally believed that regeneration from callus can cause genetic instability in the form of somaclonal variation (eg. Karp and Bright 1985) whereas regeneration from meristems does not. However there is little or no experimental evidence in the literature to support the latter. Thus a comparison of plants regenerated from callus with plants regenerated from intact meristems (micropropagation) has been carried out in order to answer this question.

Considering the restriction fragment data (Chapter 5) and the field morphology data (Chapter 8), the results of this work support the widely held view that plants regenerated from intact meristems are more stable than those regenerated from undifferentiated callus. The fact that no variant clones were identified in any of the populations derived from meristems (micropropagation and the two storage regimes) suggests that application of such techniques does not cause instability. However, it is not possible to state that there is no variability because the analyses were not complete and the number of plants analysed especially from

the storage regimes was not large. Furthermore, it is unlikely that any method of vegetative propagation of plants will be completely stable in this respect. It is well known that variants can be found even after conventional field multiplication. In this study, however, all examples of individual variant plants were found in the population of callus-derived regenerants, which further supports the theory that somaclonal variation results from a phase of undifferentiated cell proliferation.

Thus, within the constraints of time and manpower available, the original aim of the project - to assess the genetic stability of potato *in vitro* - has been achieved. Significant differences in effect between different *in vitro* regimes have been found, and correlated with regeneration either from intact meristems, or from undifferentiated callus. This difference between regeneration from meristems or from callus, emphasises the importance of the correct choice of *in vitro* regime for different purposes, and the need to be certain of the type of regeneration that is occurring. In Chapter 3 it was shown that basal callus can form at the wound site in nodal cultures (Figure 3.1) and callus can also form in other meristematic culture regimes. Adventitious shoot regeneration from this callus is possible under certain conditions, and such adventitious shoots are likely to be more variable than the shoots regenerating from intact meristems. Great care must be taken to avoid the incorporation of such adventitious shoots into the multiplication or storage regimes, either by ensuring that such shoots do not regenerate from the callus or by checking to be cer-

tain that the shoots used for further subculture originate from pre-existing meristems.

8.2 Methods of assessment

A further aim of the project was to develop and to compare different methods of analysis of plants regenerated from *in vitro* regimes. In this respect, the results presented here show that both restriction fragment analysis and field morphology analysis are suitable for this purpose, but that soluble tuber protein electrophoresis is less so. However, this latter aspect needs further consideration, because the results obtained do not show the whole picture.

8.2.1 Restriction fragment analysis

The restriction fragment analysis used in this project is certainly applicable to the study of stability *in vitro*. Although the analysis itself is technically involved and relatively expensive to carry out, it can be applied directly to tissue from plants still in culture. This aspect is particularly significant, because the other techniques are based on phenotype which require a much longer time to prepare the plant material before analysis. The techniques of restriction fragment analysis developed and used for this study are generally applicable to any analysis of restriction fragment lengths, not only for the purpose of assessing genetic stability *in vitro*. The incorporation of new methods and highly developed products has resulted in savings of both time and money, and these are expected to

continue in the future. The latest developments in membranes and blotting equipment will further speed up the transfer of DNA to filters, and - driven by the interest in RFLP mapping - probes from other species will become available (e.g. tomato - Bonierbale et al 1988). Using mixtures of low copy number probes in a 'hybridisation cocktail' (Young et al 1988) can also result in savings of time and cost.

8.2.2 Field morphology analysis

Analysis of field morphology was also useful in this study. Although only 3 variant individuals were identified compared with the 7 identified by restriction fragment analysis, significant differences were found between populations from different *in vitro* regimes. It is also probable that, had the planting material for the field trial been more consistent and thus more plants been scored under identical conditions, more individual variants could have been identified in the field. This type of analysis will continue to be very important wherever *in vitro* manipulations are used for the production of field planting material, the more so in potato as, currently, varietal identification is based on morphological characters (NIAB 1975).

8.2.3 Soluble tuber protein analysis

The problem of producing uniform planting material for analysis was probably the cause of the comparative failure of the protein analysis to yield useful results. This

analysis would probably have been of greater value if it had been carried out on the field grown tubers from the field trial. It would be expected that tubers produced under field conditions would have similar maturity and, being grown under identical conditions, produce equivalent protein spectra in gel electrophoresis. The method of tuber protein analysis is widely and usefully applied for cultivar identification (Stegemann and Schnick 1985), and so it could be expected to produce meaningful data on stability in culture.

8.2.4 Summary

In summary, it is suggested that restriction fragment analysis is quicker to carry out on plants from *in vitro* culture, but more plants could probably have been assessed using phenotype-based methods (i.e. field morphology and protein analysis of field-grown tubers) given sufficient time to perform the analyses. On the other hand, although restriction fragment analysis is relatively expensive and more applicable to smaller numbers of plants, it can be used to produce a far more complete picture of the extent of genetic variability present. An equally important point, is that it can also provide information on the basis of the variation and thus its causes.

From the preceding discussion, it can be argued that the methods are complementary and not mutually exclusive, with different methods of assessment being more useful than others in different situations. For rapid assessment of different *in vitro* regimes, or a more detailed look at stock

clones used as the mother plants for micropropagation, restriction fragment analysis would be most suitable. Alternatively, for a long term study on the effect of *in vitro* treatments using many plants, field morphological assessment is perhaps better suited, because not only can more individual plants be examined but also the nature of many of the characters scored lend themselves to a statistical approach of analysis of variance.

This complementarity is borne out by the fact that, of the individual variants identified by both restriction fragment analysis and field morphological analysis, only one clone - VA5 - was identified as variant by both analyses. Although the field trial results were not complete (some plants were not scored) this shows that analysing variation with one method alone is not sufficient to identify all variants in a population.

At present, potato cultivar identification is based on phenotypic characters (morphology - e.g. NIAB 1975 and tuber proteins - e.g. Stegemann and Schnick 1985) which argues strongly for the retention of these tests for stability assessment. However, it is envisaged that, in the not too distant future, restriction fragment analysis will be much more widely used, both for identification purposes and in breeding. As the techniques become more widespread (and developed further) it is highly likely that restriction fragment analysis will be applied more widely in assessment of *in vitro* shoots as well as those regenerated from such culture.

8.3 Role of stability testing

The procedures described in this thesis can be applied to assess the effect on genetic stability of a particular treatment, in so much as variant plants can be identified in a population of clones and variability between groups of plants produced by different *in vitro* regimes can be compared. This fulfils the major aim of the study, however, the application of such analysis (when such assessment is desirable) also needs to be considered. This is attempted in the following section, with reference to the uses of *in vitro* manipulation within potato cultivation.

8.3.1 Seed tuber multiplication schemes

The molecular analysis of stability clearly could not be carried out on all plants regenerated via *in vitro* techniques for whatever purpose. Micropropagation is now widely applied and is used (to a greater or lesser degree) for almost all seed tuber multiplication, especially in the developed world where virus elimination by meristem culture has been routinely practised for many years. Although the trend is towards reducing the number of field generations required for seed tuber production, almost all plants will at some stage be grown in the field before distribution, and so removal of non-type plants - based on field morphology - is possible. A minor degree of variation in the final crop can be tolerated, especially in less mechanised farming where a uniform crop is not as important for efficient

harvesting. In fact, a heterogenous crop can be beneficial in low-input farming systems, being less likely to succumb entirely to a single pest or environmental stress.

There is thus no requirement for absolute stability for seed tuber multiplication regimes, and so testing for stability is not a necessity. Nevertheless, excessive variation within a multiplication regime would affect seed producers as they would be forced to reject a large proportion of their crop, or risk complaints from their consumers. Knutson (1988) notes that as product quality increases, so does consumer expectation and the seed producer, often charging a premium for better quality seed tubers, must maintain standards. A little variation may be tolerable, but at least some form of 'quality control' testing within the multiplication programme is necessary. This is incorporated at present, but confined to testing for presence of virus. As fewer field generations are required (reducing the chance of virus infection) the emphasis may switch from this, to checking the genetic make-up of the population, perhaps as much to ensure correct variety labelling as to test for variation. The importance of this was illustrated at the Department of Agriculture and Fisheries for Scotland (DAFS) in 1987 (B D Hall personal communication). *In vitro* stored clones, grown in the field prior to release to seed producers, were found to be highly variable in some morphological characters. Although this was restricted to a small proportion of the clones, in some cases the entire progeny of certain mother plants was variant. Although variation to this extent was not observed the following

year, it does not detract from the observation that methods to test the genotype of the *in vitro* plants would be very useful to determine the cause of this problem, whether it was due to mis-labelling, physiological conditions or true genetic variation, for example, as a result regeneration of shoots from basal callus.

8.3.2 Germplasm storage

When wild species and primitive cultivars are maintained for potential future use in breeding, then genetic stability is very important (Withers 1987). The potato is a highly heterozygous crop, relying mainly on vegetative reproduction. Thus phenotypic adaptation will be slow and it will depend on much additive gene interaction, more so than for sexually reproducing species where there will be much more genetic flux and greater variation within populations. This makes it important to retain not only the novel genes from wild species, but also the particular combinations of those genes which are shown to be beneficial. To do this, variation within the storage regime must be reduced to a minimum (eliminating variation completely is unlikely), and so storage regimes must be assessed for their suitability on this basis.

Maintaining the additive gene interactions in breeding lines is becoming increasingly important for a novel potato multiplication scheme. The use of true (botanical) potato seed is seen as a method of solving many problems associated with the increased use of potatoes in the developing world

(Pallais 1987). The selection of good parents for the production of both hybrid and open pollinated progenies will be based on the combining abilities of these parents and so rely on the heterozygosity and additive gene interactions within the lines. It follows that variability within these lines must then be kept to a minimum in order to maintain the value of the lines as parents.

8.3.3 Genetic manipulation

In vitro culture is required for genetic manipulation of potato, both for the manipulations themselves and for the subsequent regeneration of whole plants. Since this is based on regeneration from single cells or callus, some somaclonal variation will be generated as well as the alterations caused by the inserted gene or genes. Although this may not be important to the final outcome (because the engineered plants will almost certainly need further breeding and selection before any use is made of them), it could be important, for example, to be able to separate the effect of the inserted genetic material from any somaclonal variation. It is, certainly, very important to be able to identify transgenic plants unequivocally, and so the selection system must be able to separate somaclonal variants from truly transformed material. Thus, while 'testing for stability' is not required in this case, using restriction fragment analysis to identify the transformed plants by hybridisation with probes specific to the inserted DNA is the method of choice.

Another form of genetic manipulation becoming widely used in potato breeding is somatic hybridisation (the hybridisation by fusion of somatic rather than gametic cells *in vitro*). As for transformed plants, the identification of the desired product may be made difficult by somaclonal variation within the population of regenerant plants. The technique of somatic hybridisation by mass protoplast fusion, when most efficiently applied, can produce one quarter to one third heterokaryons among a population of non-hybrid cells (Templaar and Jones 1985). Following regeneration, hybrid plants have mainly been selected on the basis of isozyme markers and shoot phenotype (eg. Foulger 1987, Fish et al 1988a), both of which are liable to be altered by somaclonal variation. The restriction fragment analysis developed in this thesis for the study of stability, has been applied to the problem of identification of hybrids and been shown to be very useful (Fish 1988, Fish et al 1988b, Pehu et al 1989). Allowing for the effect of somaclonal variation on restriction fragment patterns, the analysis is flexible enough for identification of true hybrids by the presence of hybridising bands specific to both parents.

8.3.4 Somaclonal variation

The study of stability in culture is effectively the same as the study of somaclonal variation and the methods used will thus be identical. Most work in this area has been done using phenotypic assessment, but now more groups are using molecular approaches to assess the genetic variation produced, especially where the emphasis is on the mechanism

of the induced mutation. Many different effects of the tissue culture cycle on the genome have now been documented. Amplification and reduction in copy number of repeated sequences has been shown (e.g. Landsmann and Uhrig 1985, Zheng et al 1987), single base changes have been observed (Brettel et al 1986) and increased movement of transposons also implicated (Groose and Bingham 1986).

In this work examples of altered copy number of ribosomal genes have been found in 4 cases. Complete loss of individual bands has been observed, which could be explained by deletion of fragments of DNA or by loss of complete chromosomes. The appearance of two new bands (of low copy number) in one plant could perhaps best be explained by a point mutation which created a new recognition site for the particular restriction enzyme, although this is not certain. These examples are consistent with the published examples mentioned above, and support the idea of a genetic stress being applied to the cells in culture, which results in errors of DNA replication. A number of groups are following this approach of restriction fragment analysis to study somaclonal variation at the molecular level, and more examples such as these are expected to give information on the mechanism of this phenomenon.

8.4 Conclusions

In vitro manipulations - used for genetic manipulation as well as seed tuber production - hold great potential for the improvement of potato cultivation, especially in the

developing world. However, some of these treatments can cause genetic variation which may not be apparent during *in vitro* culture, and which may affect the final regenerated plants. Methods have been developed to study such genetic variation and to compare the *in vitro* treatments for their effect on stability, as well as to identify some variant plants. It follows that when using *in vitro* approaches, the importance of maintaining genetic stability while in culture must be considered, and the particular method of culture should then be chosen to complement this. It is especially important to ensure that a callus phase is avoided if regeneration of true-to-type plants are required, even in nodal shoot cultures (used for micropropagation) basal callus is produced and care must be taken to ensure that shoot regeneration is from the meristem and not from this callus.

There are many applications of restriction fragment analysis in the field of potato improvement and cultivation, and these can be expected to be much more widely used in the future. Using a set of probes isolated from tomato (Bernatsky and Tanksley 1986) an RFLP linkage map of potato has been produced much more rapidly than expected (Bonierbale 1988). Using the techniques of pulsed field electrophoresis (Knight 1989) and yeast artificial chromosomes (Burke et al 1987), physical mapping of chromosomes is being attempted (Ganal et al 1989) which, it is hoped, will lead to the cloning of genes for many characters previously thought to be too difficult to identify (genes for polygenic characters, for example).

The study of stability in culture is only one application of restriction fragment analysis to potato improvement, nevertheless the question of the genetic stability (and variability) of potato *in vitro* is an important one and deserves attention. When this particular study was initiated, little work was being done on this problem and almost nothing had been published. However, this aspect is now changing and several groups are working on this subject, two, at least, using a restriction fragment analysis approach applied to the study of variation in potato plants regenerated from *in vitro* storage (W Powell and K Harding, personal communications). It is hoped that this thesis will provide a basis for other groups to consider the problem, and to take the work further.

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Appendix I Media recipes and solutions used

Tissue Culture Media

1/Shoot Propagation Medium

4.71	g/l	MS salts
20	g/l	Sucrose
0.1	g/l	Casein hydrolysate
0.05	mg/l	BAP
7	g/l	Agar

2/Rooting Medium

4.71	g/l	MS salts
20	g/l	Sucrose
0.05	mg/l	NAA
7	g/l	Agar

3/Liquid Culture Medium

4.71	g/l	MS salts
20	g/l	Sucrose
0.5	mg/l	BAP
0.01	mg/l	NAA
0.4	mg/l	GA ₃
2	mg/l	Calcium Pantothenate

4/Tuber Induction Medium

4.71	g/l	MS salts
80	g/l	Sucrose
5	mg/l	BAP
500	mg/l	CCC

5/Callus Induction Medium

4.71	g/l	MS salts
30	g/l	Sucrose
2.25	mg/l	BAP
0.186	mg/l	NAA
7	g/l	Agar

6/Shoot Regeneration Medium

4.71	g/l	MS salts
30	g/l	Sucrose
2.25	mg/l	BAP
5	mg/l	GA ₃
7	g/l	Agar

7/Storage Medium

4.71	g/l	MS salts
30	g/l	Sucrose
40	g/l	Mannitol
7	g/l	Agar

All media made to pH 5.8 with KOH and autoclaved at 121°C for 20 minutes. GA₃ and CCC were added after autoclaving, from filter sterilised stock solutions.

Bacterial Growth Media

1/L-Broth

5 g/l	NaCl
5 g/l	Yeast Extract
10 g/l	Bacto-tryptone

2/L-Plates

5 g/l	NaCl
5 g/l	Yeast Extract
10 g/l	Bacto-tryptone
7 g/l	Agar

3/F-Plates

5 g/l	NaCl
5 g/l	Yeast Extract
10 g/l	Bacto-tryptone
5 %	Glycerol
7 g/l	Agar

All media made to pH 7.5 with NaOH before autoclaving at 121°C for 20 minutes. Appropriate antibiotics were added from filter sterilised stock after autoclaving.

Molecular Biology Stock Solutions

All solutions made up to one litre with distilled, de-ionised water. (Maniatis et al 1983 unless stated)

1M Tris.Cl

121.1g Tris base, made to desired pH with conc.HCl.

0.5 EDTA pH 8.0

186.1g EDTA (disodium salt) approx 20g NaOH (to pH 8.0).

5M SALT

292g NaCl.

20xSSC

175.32g NaCl, 88.23g tri-sodium citrate.

20xSSPE

174g NaCl, 27.6g NaH₂PO₄.2H₂O, 7.4g EDTA, pH 7.4 (10N OH).

10xTBE

108g Tris, 55g Boric acid, 40ml 0.5M EDTA.

50xTAE

242g Tris, 57.1ml glacial acetic acid, 100ml 0.5M EDTA.

10x Electro-transfer buffer

26.65g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 28.66g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$.

5M Potassium acetate

490.7g Potassium acetate.

3M Sodium acetate

408.1g Sodium acetate, pH 5.2 with glacial acetic acid.

Denaturing solution

20 g NaOH, 87 g NaCl

M Sodium phosphate

179.11 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ pH to 7.2 with 85% orthophosphoric acid.

All above autoclaved for 20 minutes at 121°C.

20% SDS

200g Sodium dodecyl sulphate, pH 7.2 with HCl.
Filtered through Whatman No1 before use.

RNAse

10mg/ml RNAse A, boiled in water bath for 10 minutes and allowed to cool to room temperature before being stored at -20°C.

Pronase

10 mg/ml Pronase(BDH), incubated at 37°C for 2 hours before being stored at -20°C.

100x Denhardtts

20g Ficoll 400 000, 20g Poly-vinyl pyrrolidone 360,000, 20g BSA. Filtered through Nalgene .45 micron membrane before use. Stored at -20°C.

SS DNA

10mg/ml Salmon sperm DNA, sheared and boiled for 10 minutes before use. Stored at -20°C.

EtBr

10mg/ml Ethidium bromide, stored at room temperature in dark bottle using double containment.

Phenol

Distilled, saturated with M Tris.Cl pH8.0, Stored at -20°C.

Spermidine trihydrochloride

30 mg of spermidine trihydrochloride in 1 ml of sdw kept at -20°C

50% Caesium chloride

50 g Caesium chloride in 50 ml SDW

Other Molecular Biology Solutions

DNA extraction buffer I (B D Hall personal communication)

100mM NaCl
100mM Tris.Cl pH 8.0
50mM EDTA
2% SDS
0.7% 2-mercaptoethanol
200µg/ml Pronase

DNA extraction buffer II (Dellaporta et al 1983)

500 mM NaCl
100 mM Tris.Cl pH 8.0
50 mM EDTA
0.6% 2-mercaptoethanol

TE

10mM Tris.Cl pH 8.0, 1mM EDTA.

50 TE

50mM Tris.Cl pH8.0, 10mM EDTA

STE

100mM NaCl, 10mM Tris.Cl pH 8.0, 1mM EDTA.

Glycerol loading buffer

2 ml 0.5M EDTA, 3 ml SDW, 5 ml glycerol

Glucose - TE

50mM Glucose, 25mM Tris.Cl pH 8.0, 10mM EDTA

Lysozyme solution I

10.8 mg ml⁻¹ lysozyme in glucose TE
(made up just before use and stored on ice)

Lysozyme solution II

2 mg ml⁻¹ lysozyme in glucose TE
(made up just before use and stored on ice)

Alkaline SDS

SDS in NaOH

Neutralising solution

60 ml 5M Potassium acetate, 28.5 ml glacial acetic acid, 11.5 ml SDW

Sodium acetate/Tris

0.1 M Sodium acetate, 50mM Tris.Cl pH 8.0

Pre-hybridisation solution

5xSSPE
5x Denhardtts solution
0.2% SDS
200 µg/ml sheared, denatured SS DNA

Hybridisation solution is the same as above containing denatured probe solution

Church buffer (Church & Gilbert 1983)

1% BSA (crystalline grade), 1 mM EDTA, 0.5 M sodium phosphate pH 7.2, 7% SDS

Church wash solution

0.5% BSA (fraction V), 1 mM EDTA, 50 mM sodium phosphate pH 7.2, 5% SDS

Radioactive labelling solutions

(Feinberg & Vogelstein 1983)

Solution O

1.25M Tris.Cl pH 8.0, 0.125M $MgCl_2$.

Solution A

940 μ l Solution O
5 μ l 0.1M dCTP
5 μ l 0.1M dGTP
5 μ l 0.1M dTTP
18 μ l 2-ME

Solution B

2M HEPES pH 6.6

Solution C

Hexa-deoxyribo-nucleotides dissolved in TE at 90 OD units per ml.

OLB

50 Solution A : 125 Solution B : 75 Solution C - by volume. Stored at -20°C

Tuber protein electrophoresis solutions

(Stegemann & Loeschke 1976)

Extraction buffer

10 g Na_2SO_3 , 7.5 g $Na_2S_2O_5$, 50 ml SDW

Sucrose loading buffer

0.1 mg ml^{-1} Amido black, 1 g ml^{-1} Sucrose

8 x Running buffer

30.3 g Tris, 36.6 g Boric acid, made to 1 litre pH 7.9 with KOH

Acrylamide stock

28.3 g Acrylamide, 1.5 g Bis-acrylamide, 500 ml diluted running buffer

Gel solution

200 ml Acrylamide stock (de-gassed), 75 mg Na_2SO_3 , 0.5 ml DMAP, 3.5 ml freshly prepared ammonium persulphate solution (0.5 g in 25 ml water)

1% Coomassie blue

1 g Coomassie brilliant blue R in 100 ml water, filtered before use

Protein stain

25 ml 1% Coomassie blue, 30 g TCA, 200 ml methanol, 70 ml Glacial acetic acid, 800 ml water

Destain solution

300 ml methanol, 50 ml lacial acetic acid, 700 ml water

Appendix II Restriction fragment analysis data

Plant name and filter name are as given in Chapter 3.

1E, 1H, 2E etc. denotes probe/enzyme combination -

1 = SS12	B = BamHI
2 = SS15	E = EcoRI
3 = pGMO1	H = HindIII
4 = 4324	X = XbaI

+ = normal pattern
- = no data
X = variant pattern

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Appendix II
Restriction fragment analysis data

Grouped by filter

	1E	1H	2E	2H	3E	3H	4E	4H
** Filter name AB I								
A2111.1	+	+	+	+	+	+	+	+
A2113.3	+	+	+	+	+	+	+	+
A2123.1	+	+	+	+	+	+	+	+
A2511.2	+	+	+	+	+	+	+	+
A2512.1	+	+	+	-	-	+	+	+
A2521.1	+	+	+	+	+	+	+	+
A63111.1	+	+	+	+	+	+	+	+
A63111.2	+	+	+	+	+	+	+	+
A63321.1	+	+	+	+	+	+	+	+
A63322.2	+	+	+	+	+	+	+	+
A63322.3	+	+	+	+	+	+	+	+
B1211.1	+	+	+	+	+	+	+	+
B1211.2	+	+	+	+	+	+	+	+
B1222.1	+	+	+	+	+	+	+	+
B1222.2	+	+	+	+	+	+	+	+
B15231.1	+	+	+	+	+	+	+	+
B1531.1	+	+	+	+	+	+	+	+
B1533.1	+	+	+	+	+	+	+	+
B1533.2	+	+	+	+	+	+	+	+
B4222.1	-	-	-	-	-	-	-	-
B4222.2	+	+	+	+	+	+	+	+
B4231.2	+	+	+	+	+	+	+	+
B4232.1	+	+	+	+	+	+	+	+
B42321.2	+	+	+	+	+	+	+	+
B4432.2	+	+	+	+	+	+	+	+

Appendix II
Restriction fragment analysis data

Grouped by filter

	1E	1H	2E	2H	3E	3H	4E	4H
** Filter name AB II								
A2111.2	+	+	+	+	+	+	+	+
A2113.2	+	+	+	+	+	+	+	+
A2511.1	+	+	+	-	+	+	+	-
A2512.2	+	+	+	+	+	+	+	+
A63112.1	+	+	+	+	+	+	+	+
A63112.3	+	+	+	+	+	+	+	+
A63312.1	+	+	+	+	+	+	+	+
A63321.2	+	+	+	+	+	+	+	+
B1221.2	+	+	-	+	+	+	+	+
B1522.2	+	+	+	-	+	-	+	-
B15231.2	+	+	+	+	+	+	+	+
B1531.2	+	+	+	+	+	+	+	+
B4231.1	+	-	+	-	+	-	+	-
B4413.2	+	+	+	-	+	+	+	-
B4431.1	+	+	+	+	+	+	+	+

Appendix II
Restriction fragment analysis data

Grouped by filter

	1E	1H	2E	2H	3E	3H	4E	4H
** Filter name CD I								
C3313.2	+	+	+	-	+	+	+	+
C3332.1	+	+	+	+	+	+	+	+
C3332.2	+	-	+	-	+	-	+	-
C3333.1	+	+	+	+	+	+	+	+
C3333.2	+	+	+	+	+	+	+	+
C6413.2	+	+	+	+	+	+	+	+
C6432.1	+	+	+	+	+	+	+	+
C6512.2	+	-	+	-	+	-	+	-
D3111.2	+	+	+	+	+	+	+	+
D3113.2	+	+	+	+	+	+	+	+
D3512.1	+	+	+	-	-	+	-	-
D3523.1	+	+	+	+	+	+	+	+
D5223.1	+	+	+	+	+	+	+	+
D52241.1	+	+	+	+	+	+	+	+
D52242.1	+	+	+	+	+	+	+	+
D52242.2	+	+	+	+	+	+	+	+
D5432.2	+	+	+	+	+	+	+	+
D5433.2	+	+	+	-	-	+	+	-

Appendix II
Restriction fragment analysis data

Grouped by filter

	1E	1H	2E	2H	3E	3H	4E	4H
** Filter name CD II								
C3312.1	+	+	+	-	+	+	+	-
C3313.1	+	+	+	-	+	+	+	+
C3511.2	+	+	+	-	+	+	+	+
C3513.2	+	+	+	-	+	+	+	+
C3521.1	+	+	+	+	+	+	+	+
C3521.2	+	+	+	+	+	+	+	+
C3522.1	+	+	+	+	+	+	+	+
C6411.2	+	+	+	+	+	+	+	+
C6432.2	+	+	+	+	+	+	+	+
C6512.1	+	+	+	+	+	+	+	+
C6513.1	+	+	+	+	+	+	+	+
C6513.2	+	+	+	+	+	+	+	+
C6521.1	+	+	+	+	+	+	+	+
C6521.2	+	+	+	+	+	+	+	+
D3121.2	+	+	+	+	+	+	+	+
D3123.1	+	+	+	+	+	+	+	+
D3123.2	+	+	+	+	+	+	+	+
D3512.2	+	+	+	+	+	+	+	+
D3521.1	+	+	+	+	+	+	+	+
D5221.2	+	+	+	+	+	+	+	+
D52241.2	+	+	+	+	+	+	+	+
D5412.1	+	+	+	+	+	+	+	+
D5412.2	+	+	+	+	+	+	+	+
D5413.1	+	+	+	+	+	+	+	+
D5432.1	+	+	+	+	+	+	+	+

Appendix II
Restriction fragment analysis data

Grouped by filter

	1E	1H	2E	2H	3E	3H	4E	4H
** Filter name EH I								
E1122.2	+	+	+	+	+	+	+	+
E1123.2	+	-	-	-	-	-	+	-
E1131.1	+	+	+	+	+	+	+	-
E1533.1	+	+	+	+	+	+	+	+
E1533.2	+	+	+	+	+	+	+	+
E5223.2	-	+	-	-	-	-	+	-
E5232.1	+	+	+	+	+	+	+	+
E5311.1	+	+	+	+	+	+	+	+
E5311.2	+	+	+	+	+	+	+	+
E5322.1	+	+	+	+	+	+	+	+
E5322.2	-	+	+	+	-	+	+	+
E5323.1	+	+	+	+	+	+	+	+
E5323.2	-	-	-	-	-	-	+	-
H1511.1	+	+	+	+	+	+	+	+
H1533.1	+	+	+	+	+	+	+	+
H1533.2	-	-	-	-	-	-	-	-
H2222.2	+	+	+	+	+	+	+	+
H2413.1	+	-	+	-	+	-	+	-
H2413.2	-	-	-	-	-	-	-	-
H2431.1	-	+	-	+	-	+	+	+
H2431.2	+	+	+	+	+	+	+	+
H2432.2	+	+	+	+	+	+	+	+
H2511.2	+	+	+	+	+	+	+	+
H2523.2	+	+	+	+	+	+	+	+

Appendix II
Restriction fragment analysis data

Grouped by filter

	1E	1H	2E	2H	3E	3H	4E	4H
** Filter name	EH	II						
E1122.1	+	+	-	-	+	+	+	-
E1123.1	+	+	-	-	+	+	+	-
E1132.1	+	+	-	-	+	+	+	-
E1522.1	+	+	-	-	+	+	+	-
E5223.1	+	+	-	-	-	+	+	-
E5232.2	+	+	-	-	+	+	+	-
E5233.1	+	+	-	-	+	+	+	-
E5312.1	+	+	-	-	-	+	+	-
E5312.2	+	+	-	-	-	+	+	-
H1113.2	+	+	-	-	-	+	+	-
H1511.2	+	+	-	-	+	+	+	-
H1532.1	+	+	-	-	+	+	+	-
H2212.2	+	+	-	-	+	+	+	-
H2222.1	+	+	-	-	+	+	+	-
H2223.2	+	+	-	-	+	+	+	-
H2411.1	+	+	-	-	+	+	+	-
H2432.2	+	+	-	-	+	+	+	-
H2513.1	-	+	-	-	-	+	-	-
H2513.2	+	+	-	-	+	+	+	-
H2521.1	+	+	-	-	+	+	+	-
H2523.1	+	+	-	-	+	+	+	-

Appendix II
Restriction fragment analysis data

Grouped by filter

	1E	1H	2E	2H	3E	3H	4E	4H
** Filter name CST								
A.L6	+	+	+	+	+	+	+	+
A.D6	+	+	+	+	+	+	+	+
A.L12	+	+	+	+	+	+	+	+
B.L6	+	+	+	+	+	+	+	+
B.D6	+	+	+	+	+	+	+	+
B.L12	+	+	+	+	+	+	+	+
C.L6	+	+	+	+	+	+	+	+
C.D6	+	+	+	+	+	+	+	+
C.L12	+	+	+	+	+	+	+	+
D.L6	+	-	+	+	+	+	+	+
D.D6	+	+	+	+	+	+	+	+
D.L12	+	+	+	+	+	+	+	+
E.L6	+	+	+	-	+	+	+	+
E.D6	+	+	+	+	+	+	+	+
E.L12	+	-	+	+	+	+	+	+
H.L6	+	+	+	+	+	+	+	+
H.D6	+	+	+	+	+	+	+	+
H.L12	+	+	+	+	+	+	+	+

Appendix II
Restriction fragment analysis data

Grouped by filter

	1E	1H	2E	2H	3E	3H	4E	4H
** Filter name OST								
SA1	+	-	-	-	-	-	+	-
SA2	+	-	-	-	-	-	+	-
SA3	+	-	-	-	-	-	+	-
SA4	+	+	-	-	-	+	+	-
SB1	+	+	-	-	-	+	+	-
SB2	+	+	-	-	-	+	+	-
SB4	+	+	-	+	-	+	+	-
SC1	+	+	-	+	-	+	+	-
SC2	+	+	-	+	-	+	+	-
SD1	+	+	-	+	-	+	+	-
SD2	+	+	+	+	-	+	+	-
SD3	+	+	-	+	-	+	+	-
SD4	+	+	+	+	-	+	+	-
SE1	+	+	-	-	-	+	+	-
SE2	+	+	+	+	-	+	+	-
SE3	+	+	-	+	-	+	+	-
SE4	+	+	+	+	-	+	+	-
SH1	+	+	+	+	-	+	+	-
SH2	+	+	+	+	-	+	+	-
SH3	+	+	+	+	-	+	+	-
SH4	+	+	+	+	-	+	+	-

Appendix II
Restriction fragment analysis data

Grouped by filter

	1B	1E	1H	1X	2B	2E	2H	2X	3B	3E	3H	3X	4B	4E	4H	4X
** Filter name = V I																
VA1	+	+	+	-	-	+	-	-	+	-	+	+	+	+	-	-
VA3	+	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-
VA4	+	+	+	-	-	+	-	-	+	-	+	+	+	-	-	+
VA5	+	+	+	+	+	+	-	+	+	-	+	+	X	-	-	+
VA8	+	+	+	-	-	+	-	-	+	-	+	+	+	+	-	+
VA9	+	+	+	-	-	+	-	-	+	-	+	-	+	+	-	-
VA10	+	+	+	-	-	+	-	-	+	-	+	+	+	+	-	+
VB1	+	+	+	-	+	+	-	-	+	-	+	-	+	+	-	+
VB2	+	+	+	-	+	+	-	+	+	-	+	+	+	+	-	+
VB4	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VB5	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VB7	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VB8	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VB9	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VB10	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VC1	-	+	+	+	-	+	-	+	-	-	+	+	-	+	-	+
VC3	+	+	+	+	+	+	-	+	+	-	+	+	+	-	-	+
VC4	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+
VC5	+	+	+	-	+	+	-	+	+	+	+	X	+	+	-	+
VC6	+	+	+	-	+	+	-	+	+	+	+	+	+	-	-	+
VC7	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VC8	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VC9	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
VC10	+	-	+	+	+	+	-	+	+	-	+	+	+	+	-	+

Appendix II
Restriction fragment analysis data

Grouped by filter

	1B	1E	1H	1X	2B	2E	2H	2X	3B	3E	3H	3X	4B	4E	4H	4X
** Filter name = V II																
VD1	+	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+
VD2	-	-	+	-	-	+	-	-	-	-	+	-	-	+	+	-
VD3	+	-	+	-	+	X	-	-	+	+	+	+	+	+	+	+
VD6	+	-	+	+	+	-	-	+	+	-	+	+	+	+	-	+
VD7	+	-	+	+	-	+	-	+	+	-	+	+	+	+	-	+
VE1	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+
VE2	+	-	+	+	-	-	-	+	+	-	+	+	+	+	-	+
VE3	+	-	+	+	-	-	-	+	+	+	+	+	+	+	-	+
VE5	+	-	+	+	+	-	-	+	+	+	+	+	+	+	-	+
VE6	+	-	+	+	+	-	-	+	+	-	+	+	+	+	-	+
VE8	+	-	+	+	+	-	-	+	+	+	+	+	+	+	-	+
VE10	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+
VH1	+	-	+	+	-	+	-	+	+	-	+	X	+	+	+	+
VH2	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+
VH3	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+
VH4	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+
VH5	-	-	+	+	-	+	-	+	-	+	+	+	-	+	+	+
VH6	+	-	+	+	-	+	-	+	+	-	+	+	+	+	-	+
VH7	X	-	+	+	-	+	-	+	+	+	+	+	+	X	+	+
VH8	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
VH10	+	-	+	+	+	+	-	+	+	X	+	+	X	X	+	+
VHX	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+

Appendix III Tuber protein electrophoresis data

Plant name as given in Chapter 3.

Tuber weight is in grammes.

Numbers correspond to protein bands as in Chapter 6.

+ = prescence of normal band

- = absence of band in that region of gel

2 = extra band in that region of gel

Appendix III
Protein electrophoresis data

	tuber weight	1	2	3	4	5	6	7	8	9
A2111.1	10.7	+	+	+	+	+	+	+	+	+
A2111.2	10.5	+	+	+	+	+	+	+	-	+
A2113.1	6.4	+	+	+	+	+	+	-	+	+
A2113.2	7.8	+	+	+	+	+	+	-	+	+
A2123.1	6.7	+	+	+	+	-	+	+	+	+
A2511.1	10.3	+	+	+	+	+	+	-	+	+
A2511.2	9.7	+	+	+	+	+	+	-	+	+
A2512.1	8.3	+	+	+	+	+	+	+	+	+
A2512.2	5.5	+	+	+	+	+	+	+	+	+
A2521.1	7.8	+	+	+	+	+	+	+	+	+
A63111.1	9.2	+	+	+	+	+	+	+	+	+
A63111.2	9.6	+	+	+	+	-	+	+	+	+
A63112.1	8.0	+	+	+	+	+	+	+	+	+
A63112.3	5.9	+	+	+	+	-	+	-	+	+
A63312.1	10.8	+	-	+	-	-	+	-	+	+
A63321.1	5.4	+	+	+	+	+	+	+	+	+
A63321.2	7.2	+	+	+	+	+	+	+	+	+
A63322.2	10.4	+	+	+	+	+	+	+	+	+
A63322.3	8.7	+	+	+	+	+	+	+	+	+
B1211.1	15.8	+	+	+	+	+	+	+	+	+
B1211.2	4.8	+	+	+	+	+	+	-	+	+
B1213.1	23.1	+	+	+	+	+	+	-	+	+
B1221.1	6.1	+	+	+	+	+	+	+	+	+
B1221.2	8.4	+	+	+	+	-	+	+	+	-
B1222.1	3.3	+	+	+	+	-	2	-	+	+
B1222.2	8.8	+	+	+	+	-	+	+	+	+
B1522.2	3.3	+	+	+	+	+	2	-	+	+
B15231.1	6.7	+	+	+	+	+	+	-	+	+
B15231.2	4.9	+	+	+	+	+	2	-	+	+
B1531.1	5.2	+	+	+	+	+	+	+	+	+
B1531.2	4.5	+	+	+	+	+	+	-	+	+
B1533.1	7.4	+	+	+	+	+	+	+	+	+
B1533.2	6.1	+	+	+	+	+	+	+	+	+
B4222.1	8.3	+	+	+	+	+	+	+	+	+
B4222.2	15.6	+	+	+	+	-	+	+	+	+
B4231.1	3.5	+	+	+	+	+	+	-	+	+
B4231.2	23.9	+	+	+	+	+	+	-	+	+
B42321.1	8.0	+	+	+	+	+	+	+	+	+
B42321.2	3.4	+	+	+	+	+	+	+	+	+
B4413.2	7.4	+	+	+	+	+	+	+	+	+
B4431.1	4.0	+	+	+	+	+	+	+	+	+
B4432.2	13.2	+	+	+	+	+	+	-	+	+
C3312.1	9.6	+	+	+	+	-	+	-	+	+
C3312.2	8.6	+	+	+	+	-	+	+	+	+
C3313.1	4.6	+	+	+	+	-	+	-	+	+
C3313.2	12.6	+	+	+	-	-	+	-	+	+
C3332.1	9.7	+	+	+	+	+	+	-	+	+

Appendix III
Protein electrophoresis data

	tuber weight	1	2	3	4	5	6	7	8	9
C3332.2	11.1	+	+	+	+	-	+	-	+	+
C3333.1	8.0	+	+	+	-	-	+	-	+	+
C3333.2	14.3	+	+	+	+	-	+	+	+	+
C3511.2	6.7	+	+	+	+	-	+	+	+	+
C3513.2	5.0	+	+	+	+	-	+	+	+	+
C3521.1	6.9	+	+	+	+	-	+	-	+	+
C3521.2	3.9	+	+	+	+	-	+	-	+	+
C3522.1	5.6									
C6411.2	6.7	+	+	+	+	+	+	-	+	+
C6413.2	11.6	+	+	+	+	+	+	-	+	+
C6432.1	6.3	+	+	+	+	-	+	-	+	+
C6432.2	4.1	+	+	+	+	-	+	+	+	+
C6512.1	5.6	+	+	+	+	+	+	-	+	+
C6512.2	5.7	+	+	+	+	-	+	+	+	+
C6513.1	4.1	+	+	+	+	-	+	+	+	+
C6513.2	3.6	+	+	+	+	-	+	+	+	+
C6521.1	3.8	+	+	+	+	+	+	-	+	+
C6521.2	5.8	+	+	+	+	+	+	-	+	+
D3111.2	4.5	+	+	+	+	+	+	-	+	+
D3113.2	12.4	+	+	+	+	+	+	-	+	+
D3121.2	5.0	+	+	+	+	+	+	+	+	+
D3123.1	5.4	+	+	+	+	-	+	+	+	+
D3123.2	3.9	+	+	+	+	+	+	-	+	+
D3512.1	4.2	+	+	+	+	+	+	+	+	+
D3512.2	5.1	+	+	+	+	-	+	+	+	+
D3521.1	3.5	+	+	+	+	-	+	+	+	+
D3523.1	10.3	+	-	+	+	-	+	-	+	+
D3523.2	7.6	+	+	+	+	+	+	+	+	+
D5221.2	8.2	+	+	+	+	-	+	+	+	+
D5223.1	3.1	+	+	+	+	-	+	-	+	+
D52241.1	8.5	+	+	+	+	-	+	-	+	+
D52241.2	4.8	+	+	+	+	+	+	-	+	+
D52242.1	12.8	+	+	+	-	-	+	-	+	+
D52242.2	9.9	+	+	+	+	-	+	-	+	+
D5412.1	5.3	+	+	+	+	-	+	+	+	+
D5412.2	2.8	+	+	+	+	-	+	+	+	+
D5413.1	3.4	+	+	+	+	-	+	+	+	+
D5432.1	5.7	+	+	+	+	+	+	-	+	+
D5432.2	6.8	+	+	+	+	+	+	-	+	+
D5433.2	13.4	+	+	+	+	+	+	-	+	+
E1122.1	7.3	+	+	+	+	+	+	-	+	+
E1123.1	5.5	+	+	+	+	+	+	-	+	+
E1123.2	12.0	+	+	+	+	+	+	-	+	+
E1131.1	13.3	+	+	+	+	-	+	+	+	+
E1132.1	3.0	+	+	+	+	-	+	+	+	+
E1522.1	3.0	+	+	+	+	+	+	-	+	+
E1533.1	17.1	+	+	+	+	+	+	-	+	+

Appendix III
Protein electrophoresis data

	tuber weight	1	2	3	4	5	6	7	8	9
E1533.2	12.8	+	+	+	+	+	+	-	+	+
E5223.1	7.1	+	+	+	+	+	+	-	+	+
E5223.2	8.0	+	+	+	+	+	+	+	+	+
E5232.1	8.9	+	+	+	+	+	+	-	+	+
E5232.2	3.3									
E5233.1	7.1	+	+	+	+	+	+	-	+	+
E5311.1	6.1	+	+	+	+	-	+	-	+	+
E5311.2	11.7	+	+	+	+	-	+	-	+	+
E5312.1	5.2	+	+	+	+	-	+	+	+	+
E5312.2	4.5	+	+	+	-	-	+	-	+	+
E5322.1	11.2	+	+	+	+	+	+	-	+	+
E5322.2	10.1	+	+	+	+	+	+	-	+	+
E5323.1	7.3	+	+	+	+	+	+	-	+	+
E5323.2	11.4	+	+	+	+	+	+	+	+	+
H1113.2	5.7	+	+	+	+	-	+	-	+	+
H1511.1	15.0	+	+	+	+	-	+	+	+	+
H1511.2	7.7	+	+	+	+	-	+	+	+	+
H1532.1	3.2	+	+	+	+	+	+	-	+	+
H1533.1	13.7	+	+	+	+	+	+	-	+	+
H1533.2	4.6	+	+	+	+	+	+	-	+	+
H2212.2	10.4	+	+	+	+	+	+	+	+	+
H2222.1	4.0	+	+	+	+	-	+	+	+	+
H2222.2	2.8	+	+	+	+	+	+	-	+	+
H2223.2	6.2	+	+	+	-	-	+	+	+	+
H2411.1	5.4	+	+	+	+	+	+	+	+	+
H2413.1	5.7	+	+	+	+	+	+	+	+	+
H2413.2	7.4	+	+	+	+	+	+	-	+	+
H2431.1	5.4	+	+	+	+	+	+	-	+	+
H2431.2	8.0	+	+	+	+	-	+	-	+	+
H2432.1	6.2	+	+	+	+	+	+	+	+	+
H2432.2	4.0	+	+	+	+	+	+	-	+	+
H2511.2	13.1	+	+	+	+	-	+	+	+	+
H2513.1	0.0	+	+	+	+	-	+	+	+	+
H2513.2	3.0	+	+	+	+	+	+	-	+	+
H2521.1	9.2	+	+	+	+	+	+	+	+	+
H2523.1	2.9	+	+	+	+	+	+	-	+	+
H2523.2	21.7	+	+	+	+	+	+	+	+	+

Appendix III Protein electrophoresis data

	tuber weight	1	2	3	4	5	6	7	8	9
SA2	6.2	+	+	+	+	+	+	+	+	+
SA4	5.8	+	+	+	+	-	+	+	+	+
SB1	6.6	+	+	+	+	+	+	-	+	+
SB2	11.9	+	+	+	+	-	+	+	+	+
SB4	11.3	+	+	+	+	-	+	-	+	+
SC2	10.3	+	+	+	-	-	+	-	+	+
SD1	7.3	+	+	+	+	-	+	+	+	+
SD2	3.0	+	+	+	+	-	+	+	+	+
SD4	6.8	+	+	+	+	-	+	+	+	+
SE1	6.2	+	+	+	+	-	+	+	+	+
SE2	9.1	+	+	+	+	-	+	+	+	+
SE3	4.8	+	+	+	+	-	+	+	+	+
SH1	5.1	+	+	+	+	-	+	+	+	+
SH2	2.9	+	+	+	+	+	+	+	+	+
SH3	4.6	+	+	+	+	+	+	+	+	+
SH4	3.2	+	+	+	+	+	+	+	+	+

Appendix III
Protein electrophoresis data

	tuber weight	1	2	3	4	5	6	7	8	9
VA1	6.6	+	+	+	+	+	+	+	+	+
VA3	8.7	+	+	+	+	+	+	-	+	+
VA4	5.1	+	+	+	+	+	+	+	+	+
VA5	7.1	+	+	+	+	+	+	-	+	+
VA8	17.7	+	+	+	+	+	+	+	+	+
VA9	5.8	+	+	+	+	+	+	-	+	+
VA10	7.0	+	+	+	+	+	+	-	+	+
VB1	6.0	+	+	+	-	-	+	+	+	+
VB2	8.8	+	+	+	+	+	+	-	+	+
VB4	10.3	+	+	+	+	+	+	+	+	+
VB5	10.3	+	+	+	+	+	+	-	+	+
VB7	4.6	+	+	+	+	+	+	-	+	+
VB8	5.4	+	+	+	+	+	+	-	+	+
VB10	7.8	+	+	+	+	+	+	-	+	+
VC3	6.8	+	+	+	+	-	+	-	+	+
VC4	7.0	+	+	+	+	-	+	+	+	+
VC5	9.8	+	+	+	+	+	+	-	+	+
VC6	3.4	+	+	+	+	+	+	-	+	+
VD1	3.9	+	+	+	+	+	+	+	+	+
VD2	2.4	+	+	+	+	-	+	+	+	+
VD6	10.0	+	+	+	+	+	+	+	+	+
VD7	8.2	+	+	+	+	+	+	+	+	+
VE1	11.5	+	+	+	+	-	+	+	+	+
VE2	5.4	+	+	+	+	-	+	+	+	+
VE3	9.3	+	+	+	+	-	+	+	+	+
VE5	11.8	2	+	+	+	-	+	+	+	+
VE6	2.9	2	+	+	+	-	+	+	+	+
VE8	9.6	2	+	+	+	-	+	+	+	+
VE10	6.5	+	+	+	+	-	+	-	+	+
VH1	3.8	+	+	+	+	+	2	-	+	+
VH2	4.9	+	+	+	+	+	+	+	+	+
VH3	7.2	+	+	+	+	+	2	-	+	+
VH7	8.9	+	+	+	+	-	+	+	+	+
VH8	7.0	+	+	+	+	-	+	+	+	+
VH10	3.9	+	+	+	-	-	-	-	+	+
VC2	6.3	+	+	+	+	+	+	+	+	+
VD5	9.5	+	+	+	+	+	+	+	+	+
VE4	7.7	2	+	+	+	+	+	+	+	+
VE9	5.7	2	+	+	+	-	+	+	+	+
VHY	6.4	+	-	-	-	-	+	-	+	+
VX1	7.9	+	+	+	+	-	+	+	+	+
VX2	14.3	+	+	+	+	-	+	+	+	+
VX3	9.2	+	+	+	+	-	+	+	+	+
VX4	8.1	+	+	+	+	+	+	+	+	+

Appendix IV Field trial data

Key to tables

Plant name and type are as in Chapter 3, replicates denoted by lower case letters following (CON = control plants). Date refers to the date on which morphology measurements were taken (harvest date for tubers not given)

Table 1. Leaf-stem characters

1. Compound leaf	1= terminal solitary 2= terminal touching laterals - not overlapped 3= terminal just overlapped - one cm 4= large overlap 5= terminal obscured by laterals
2. Terminal leaflet shape - length/width	
3. Lateral leaflet shape - length/width	
4. Stem wings	1= absent 2= straight 3= bent 4= wavy 5= very wavy
5. Anthocyanin	1= absent
a-stem	2= trace
b-leaf axil	3= mottling
c-rachides	4= patch 5= general 6= internodes only 7= intensive
6. Foliage colour	1= yellow green 2= light green 3= grey green 4= mid-green 5= dark green 6= blue green
7. Foliage gloss	1= dull 2= very light 3= light 4= medium 5= heavy 6= heavy

Table 2. Flower and tuber characters

1. Flower colour	1= purple 2= light purple 3= trace of purple 4= white
2. Anther colour	1= yellow 2= orange 3= mixed
3. Anther morphology	1= normal 2= abnormal
4. Protrusion of style	1= hidden 2= > 1 mm 3= 1 - 3 mm 4= 3 - 5 mm
5. Berries	0= absent 1= present
6. Tuber shape	1= round 2= short oval 3= oval 4= kidney 5= long oval 6= long
7. Tuber skin colour	1= white 2= pink 3= purple 4= red
8. Tuber flesh colour	1= white 2= cream 3= lemon 4= yellow

Table 3. Tuber numbers and weights
in each size class

class 1=	> 10 g
2=	10 - 30 g
3=	30 - 100 g
4=	100 - 200 g
5=	200 - 500 g
6=	< 500g

Plants with a measurement date of 27/7/88 were used for statistical analysis.

Appendix IV
Table 1. Leaf-stem characters

Grouped by plant type

		1	2	3	4	5a	5b	5c	6	7
** Plant type = CON										
A a	/ /	0	0.00	0.00	0	0	0	0	0	0
A b	/ /	0	0.00	0.00	0	0	0	0	0	0
A c	/ /	0	0.00	0.00	0	0	0	0	0	0
A d	/ /	0	0.00	0.00	0	0	0	0	0	0
B a	/ /	0	0.00	0.00	0	0	0	0	0	0
B b	/ /	0	0.00	0.00	0	0	0	0	0	0
B c	/ /	0	0.00	0.00	0	0	0	0	0	0
B d	/ /	0	0.00	0.00	0	0	0	0	0	0
C a	25/08/88	3	1.44	1.67	3	5	5	7	5	4
C b	/ /	0	0.00	0.00	0	0	0	0	0	0
C c	09/08/88	3	1.60	1.56	4	5	5	7	5	4
C d	08/09/88	3	1.41	1.62	3	5	5	7	5	4
D a	25/08/88	3	1.44	1.53	4	5	5	7	5	4
D b	09/08/88	3	1.41	1.60	3	5	5	7	5	4
D c	25/08/88	3	1.56	1.56	3	5	5	7	5	4
D d	/ /	0	0.00	0.00	0	0	0	0	0	0
E a	25/08/88	3	1.39	1.65	4	5	5	7	5	4
E b	21/09/88	3	1.46	1.43	3	5	5	7	5	4
E c	25/08/88	3	1.34	1.57	3	5	5	7	5	4
E d	/ /	0	0.00	0.00	0	0	0	0	0	0
H a	09/08/88	3	1.42	1.53	4	5	5	7	5	4
H b	25/08/88	3	1.51	1.50	3	5	5	7	5	4
H c	/ /	0	0.00	0.00	0	0	0	0	0	0
H d	09/08/88	3	1.46	1.47	3	5	5	7	5	4

Appendix IV
Table 1. Leaf-stem characters

		Grouped by plant type									
		1	2	3	4	5a	5b	5c	6	7	
** Plant type = MP											
A2111.1	08/09/88	3	1.53	1.90	3	5	5	7	5	4	
A2111.2	/ /	0	0.00	0.00	0	0	0	0	0	0	
A2113.1	08/09/88	3	1.40	1.63	3	5	5	7	5	4	
A2113.2	/ /	0	0.00	0.00	0	0	0	0	0	0	
A2123.1	/ /	0	0.00	0.00	0	0	0	0	0	0	
A2511.1	/ /	0	0.00	0.00	0	0	0	0	0	0	
A2511.2	21/09/88	3	1.51	1.69	3	5	5	7	5	4	
A2512.1	/ /	0	0.00	0.00	0	0	0	0	0	0	
A2512.2	21/09/88	3	1.56	1.76	3	5	5	7	5	4	
A2521.1	08/09/88	3	1.63	1.71	4	5	5	7	5	4	
A63111.1	/ : /	0	0.00	0.00	0	0	0	0	0	0	
A63111.2	27/07/88	3	1.56	1.53	4	5	5	7	5	4	
A63112.1	21/09/88	3	1.40	1.59	3	5	5	7	5	4	
A63112.3	08/09/88	3	1.39	1.62	3	5	5	7	5	4	
A63312.1	/ /	0	0.00	0.00	0	0	0	0	0	0	
A63321.1	27/07/88	3	1.38	1.68	3	5	5	7	5	4	
A63321.2	08/09/88	3	1.42	1.69	3	5	5	7	5	4	
A63322.2	27/07/88	3	1.55	1.40	3	5	5	7	4	4	
A63322.3	15/08/88	3	1.29	1.49	3	5	5	7	5	4	
B1211.1	09/08/88	3	1.49	1.59	4	5	5	7	5	4	
B1211.2	/ /	0	0.00	0.00	0	0	0	0	0	0	
B1213.1	27/07/88	3	1.59	1.73	3	5	5	7	5	4	
B1221.2	21/09/88	3	1.39	1.49	3	5	5	7	5	4	
B1222.1	08/09/88	3	1.41	1.72	4	5	5	7	5	4	
B1222.2	27/07/88	3	1.45	1.74	3	5	5	7	5	4	
B1522.2	/ /	0	0.00	0.00	0	0	0	0	0	0	
B15231.1	27/07/88	3	1.43	1.50	4	5	5	7	4	3	
B15231.2	/ /	0	0.00	0.00	0	0	0	0	0	0	
B1531.1	21/09/88	3	1.36	1.69	3	5	5	7	5	4	
B1531.2	08/09/88	3	1.46	1.58	3	5	5	7	5	4	
B1533.1	09/08/88	3	1.48	1.40	4	5	5	7	5	4	
B1533.2	21/09/88	3	1.38	1.64	3	5	5	7	5	4	
B4222.1	21/09/88	3	1.36	1.76	4	5	5	7	5	4	
B4222.2	27/07/88	3	1.58	1.70	3	5	5	7	5	4	
B4231.1	08/09/88	3	1.42	1.50	3	5	5	7	5	4	
B4231.2	09/08/88	3	1.37	1.33	4	5	5	7	5	4	
B42321.1	08/09/88	3	1.51	1.47	3	5	5	7	5	4	
B42321.2	08/09/88	3	1.48	1.81	3	5	5	7	5	4	
B4413.2	21/09/88	3	1.35	1.66	3	5	5	7	5	4	
B4431.1	/ /	0	0.00	0.00	0	0	0	0	0	0	
B4432.2	27/07/88	3	1.33	1.67	4	5	5	7	5	4	
C3312.1	09/08/88	3	1.47	1.33	3	5	5	7	5	4	
C3312.2	27/07/88	3	1.44	1.67	4	5	5	7	5	4	
C3313.1	/ /	0	0.00	0.00	0	0	0	0	0	0	
C3313.2	27/07/88	3	1.38	1.63	3	5	5	7	5	4	
C3332.1	27/07/88	3	1.48	1.72	3	5	5	7	5	3	
C3332.2	27/07/88	3	1.52	1.63	4	5	5	7	5	4	

Appendix IV
Table 1. Leaf-stem characters

		Grouped by plant type								
		1	2	3	4	5a	5b	5c	6	7
C3333.1	27/07/88	3	1.50	1.77	4	5	5	7	5	4
C3333.2	27/07/88	3	1.40	1.57	4	5	5	7	5	4
C3511.2	08/09/88	3	1.55	1.75	4	5	5	7	5	4
C3513.2	21/09/88	3	1.38	1.62	3	5	5	7	5	4
C3521.1	21/09/88	3	1.54	1.70	3	5	5	7	5	4
C3521.2	25/08/88	3	1.50	1.53	4	5	5	7	5	4
C3522.1	/ /	0	0.00	0.00	0	0	0	0	0	0
C6411.2	08/09/88	3	1.40	1.57	3	5	5	7	5	4
C6413.2	09/08/88	3	1.39	1.49	3	5	5	7	5	4
C6432.1	/ /	0	0.00	0.00	0	0	0	0	0	0
C6432.2	/ /	0	0.00	0.00	0	0	0	0	0	0
C6512.1	/ /	0	0.00	0.00	0	0	0	0	0	0
C6512.2	27/07/88	3	1.37	1.59	3	5	5	7	5	4
C6513.1	08/09/88	3	1.46	1.54	4	5	5	7	5	4
C6513.2	/ /	0	0.00	0.00	0	0	0	0	0	0
C6521.1	/ /	0	0.00	0.00	0	0	0	0	0	0
C6521.2	08/09/88	3	1.51	1.70	3	5	5	7	5	4
D3111.2	08/09/88	3	1.46	1.74	3	5	5	7	5	4
D3113.2	09/08/88	3	1.40	1.64	4	5	5	7	5	4
D3121.2	/ /	0	0.00	0.00	0	0	0	0	0	0
D3123.1	/ /	0	0.00	0.00	0	0	0	0	0	0
D3123.2	/ /	0	0.00	0.00	0	0	0	0	0	0
D3512.1	27/07/88	3	1.49	1.71	5	5	5	7	4	4
D3512.2	08/09/88	3	1.47	1.68	4	5	5	7	5	4
D3521.2	21/09/88	3	1.42	1.63	3	5	5	7	5	4
D3523.1	21/09/88	3	1.43	1.62	3	5	5	7	5	4
D3523.2	27/07/88	3	1.47	1.69	4	5	5	7	5	4
D5221.2	21/09/88	3	1.38	1.54	3	5	5	7	5	4
D5223.1	15/08/88	3	1.40	1.45	3	5	5	7	5	4
D52241.1	/ /	0	0.00	0.00	0	0	0	0	0	0
D52241.2	08/09/88	3	1.41	1.62	2	5	5	7	5	4
D52242.1	27/07/88	3	1.42	1.58	3	5	5	7	5	4
D52242.2	27/07/88	3	1.56	1.66	3	5	5	7	5	4
D5412.1	/ /	0	0.00	0.00	0	0	0	0	0	0
D5412.2	/ /	0	0.00	0.00	0	0	0	0	0	0
D5413.1	21/09/88	3	1.63	1.54	3	5	5	7	5	4
D5432.1	/ /	0	0.00	0.00	0	0	0	0	0	0
D5432.2	27/07/88	3	1.28	1.81	4	5	5	7	5	4
D5433.2	/ /	0	0.00	0.00	0	0	0	0	0	0
E1122.1	/ /	0	0.00	0.00	0	0	0	0	0	0
E1123.1	21/09/88	3	1.36	1.54	3	3	3	7	4	4
E1123.2	27/07/88	3	1.50	1.66	4	5	5	7	5	4
E1131.1	08/09/88	3	1.42	1.54	4	5	5	7	5	4
E1132.1	/ /	0	0.00	0.00	0	0	0	0	0	0
E1522.1	/ /	0	0.00	0.00	0	0	0	0	0	0
E1533.1	27/07/88	3	1.66	1.62	4	5	5	7	4	5
E1533.2	15/08/88	3	1.44	1.55	4	5	5	7	5	4
E5223.1	/ /	0	0.00	0.00	0	0	0	0	0	0

Appendix IV
Table 1. Leaf-stem characters

		Grouped by plant type								
		1	2	3	4	5a	5b	5c	6	7
E5223.2	27/07/88	3	1.56	1.45	4	5	5	7	5	4
E5232.1	08/09/88	3	1.53	1.53	4	5	5	7	5	4
E5232.2	21/09/88	3	1.51	1.71	3	5	5	7	5	4
E5233.1	/ /	0	0.00	0.00	0	0	0	0	0	0
E5311.1	21/09/88	3	1.40	1.45	3	5	5	7	5	4
E5311.2	27/07/88	3	1.30	1.59	4	5	5	7	4	4
E5312.1	21/09/88	3	1.38	1.76	3	5	5	7	5	4
E5312.2	21/09/88	3	1.37	1.47	3	5	5	7	5	4
E5322.1	27/07/88	3	1.52	1.67	4	5	5	7	5	4
E5322.2	15/08/88	3	1.36	1.55	4	5	5	7	5	4
E5323.1	08/09/88	3	1.43	1.68	3	5	5	7	5	4
E5323.2	27/07/88	3	1.47	1.63	3	5	5	7	5	4
H1113.2	25/08/88	0	1.38	1.36	3	5	5	7	5	4
H1511.1	09/08/88	3	1.26	1.47	4	5	5	7	5	4
H1511.2	08/09/88	3	1.51	1.53	3	5	5	7	5	4
H1532.1	/ /	0	0.00	0.00	0	0	0	0	0	0
H1533.1	/ /	0	0.00	0.00	0	0	0	0	0	0
H1533.2	27/07/88	3	1.47	1.86	4	5	5	7	5	4
H2212.2	08/09/88	3	1.51	1.63	3	5	5	7	5	4
H2222.1	08/09/88	3	1.54	1.68	3	5	5	7	5	4
H2222.2	25/08/88	3	1.52	1.48	3	5	5	7	5	4
H2223.2	08/09/88	3	1.40	1.74	3	5	5	7	5	4
H2411.1	08/09/88	3	1.45	1.76	3	5	5	7	5	4
H2413.1	08/09/88	3	1.49	1.58	3	5	5	7	5	4
H2413.2	08/09/88	3	1.45	1.74	4	5	5	7	5	4
H2431.1	27/07/88	3	1.53	1.73	3	5	5	7	5	4
H2431.2	/ /	0	0.00	0.00	0	0	0	0	0	0
H2432.1	27/07/88	3	1.55	1.87	4	5	5	7	5	4
H2432.2	/ /	0	0.00	0.00	0	0	0	0	0	0
H2511.2	27/07/88	3	1.33	1.59	4	7	5	7	5	4
H2513.1	08/09/88	3	1.46	1.67	3	5	5	7	5	4
H2513.2	/ /	0	0.00	0.00	0	0	0	0	0	0
H2521.1	15/08/88	3	1.39	1.38	3	7	5	7	5	4
H2523.1	/ /	0	0.00	0.00	0	0	0	0	0	0
H2523.2	27/07/88	3	1.50	1.66	4	5	5	7	5	4

Appendix IV
Table 1. Leaf-stem characters

Grouped by plant type			1	2	3	4	5a	5b	5c	6	7
** Plant type = CST											
A.L12 a	/	/	0	0.00	0.00	0	0	0	0	0	0
A.L12 b	/	/	0	0.00	0.00	0	0	0	0	0	0
B.D6 a	/	/	0	0.00	0.00	0	0	0	0	0	0
B.D6 b	/	/	0	0.00	0.00	0	0	0	0	0	0
B.L12 a	/	/	0	0.00	0.00	0	0	0	0	0	0
B.L12 b	/	/	0	0.00	0.00	0	0	0	0	0	0
C.D6 a	/	/	0	0.00	0.00	0	0	0	0	0	0
C.D6 b	/	/	0	0.00	0.00	0	0	0	0	0	0
C.L12 a	25/08/88		3	1.33	1.55	3	5	5	7	5	4
C.L12 b	08/09/88		3	1.40	1.62	3	5	5	7	5	4
C.L6 a	/	/	0	0.00	0.00	0	0	0	0	0	0
C.L6 b	/	/	0	0.00	0.00	0	0	0	0	0	0
D.D6 a	/	/	0	0.00	0.00	0	0	0	0	0	0
D.D6 b	/	/	0	0.00	0.00	0	0	0	0	0	0
D.L12 a	25/08/88		3	1.60	1.55	3	7	5	7	5	4
D.L12 b	/	/	0	0.00	0.00	0	0	0	0	0	0
D.L6 a	/	/	0	0.00	0.00	0	0	0	0	0	0
D.L6 b	08/09/88		3	1.36	1.58	3	5	5	7	5	4
E.D6 a	25/08/88		3	1.50	1.60	3	7	5	7	5	4
E.D6 b	/	/	0	0.00	0.00	0	0	0	0	0	0
E.L12 a	/	/	0	0.00	0.00	0	0	0	0	0	0
E.L12 b	/	/	0	0.00	0.00	0	0	0	0	0	0
E.L6 a	/	/	0	0.00	0.00	0	0	0	0	0	0
E.L6 b	/	/	0	0.00	0.00	0	0	0	0	0	0
H.D6 a	/	/	0	0.00	0.00	0	0	0	0	0	0
H.D6 b	15/08/88		3	1.25	1.49	3	5	5	7	5	4
H.L12 a	08/09/88		3	1.57	1.67	3	5	5	7	5	4
H.L12 b	/	/	0	0.00	0.00	0	0	0	0	0	0
H.L6 a	/	/	0	0.00	0.00	0	0	0	0	0	0
H.L6 b	/	/	0	0.00	0.00	0	0	0	0	0	0

Appendix IV
Table 1. Leaf-stem characters

Grouped by plant type

		1	2	3	4	5a	5b	5c	6	7
** Plant type = OST										
SA1 b	/ /	0	0.00	0.00	0	0	0	0	0	0
SA2 a	/ /	0	0.00	0.00	0	0	0	0	0	0
SA2 b	/ /	0	0.00	0.00	0	0	0	0	0	0
SA4 a	21/09/88	3	1.37	1.67	3	5	5	7	5	4
SA4 b	08/09/88	3	1.42	1.49	3	5	5	7	5	4
SB1 a	/ /	0	0.00	0.00	0	0	0	0	0	0
SB1 b	08/09/88	3	1.33	1.61	3	5	5	7	5	4
SB2 a	08/09/88	3	1.51	1.60	2	5	5	7	5	4
SB2 b	08/09/88	3	1.43	1.71	3	5	5	7	5	4
SB4 a	21/09/88	3	1.32	1.60	4	5	5	7	5	4
SB4 b	/ /	0	0.00	0.00	0	0	0	0	0	0
SC2 a	08/09/88	3	1.53	1.65	4	5	5	7	5	4
SC2 b	/ /	0	0.00	0.00	0	0	0	0	0	0
SD1 a	21/09/88	3	1.48	1.72	3	5	5	7	5	4
SD1 b	08/09/88	3	1.40	1.65	3	5	5	7	5	4
SD2 a	15/08/88	3	1.49	1.68	3	5	7	7	5	4
SD2 b	/ /	0	0.00	0.00	0	0	0	0	0	0
SD4 a	/ /	0	0.00	0.00	0	0	0	0	0	0
SD4 b	/ /	0	0.00	0.00	0	0	0	0	0	0
SE1 a	/ /	0	0.00	0.00	0	0	0	0	0	0
SE1 b	09/08/88	3	1.43	1.65	3	5	5	7	5	4
SE2 a	25/08/88	3	1.43	1.47	4	5	5	7	5	4
SE2 b	15/08/88	3	1.48	1.69	3	5	5	7	5	4
SE3 a	21/09/88	3	1.37	1.63	3	5	5	7	5	4
SE3 b	15/08/88	3	1.37	1.58	3	5	5	7	5	4
SH1 a	/ /	0	0.00	0.00	0	0	0	0	0	0
SH2 a	/ /	0	0.00	0.00	0	0	0	0	0	0
SH2 b	15/08/88	3	1.44	1.57	3	5	5	7	5	4
SH3 a	09/08/88	3	1.33	1.15	3	5	5	7	5	4
SH3 b	/ /	0	0.00	0.00	0	0	0	0	0	0
SH4 a	/ /	0	0.00	0.00	0	0	0	0	0	0
SH4 b	/ /	0	0.00	0.00	0	0	0	0	0	0

Appendix IV
Table 1. Leaf-stem characters

Grouped by plant type

		1	2	3	4	5a	5b	5c	6	7
** Plant type = REG										
VA1 a	09/08/88	3	1.38	1.68	3	5	5	7	5	4
VA1 b	27/07/88	3	1.65	2.14	3	5	5	7	5	4
VA10 a	08/09/88	3	1.60	1.89	3	5	5	7	5	4
VA10 b	/ /	0	0.00	0.00	0	0	0	0	0	0
VA3 a	15/08/88	3	1.22	1.43	3	7	5	7	5	4
VA3 b	/ /	0	0.00	0.00	0	0	0	0	0	0
VA4 a	/ /	0	0.00	0.00	0	0	0	0	0	0
VA4 b	/ /	0	0.00	0.00	0	0	0	0	0	0
VA5 a	27/07/88	3	1.60	1.73	4	5	3	7	5	4
VA5 b	09/08/88	3	1.43	1.67	4	7	5	7	5	4
VA8 a	/ /	0	0.00	0.00	0	0	0	0	0	0
VA8 b	08/09/88	2	1.33	1.68	2	5	5	7	5	4
VA9 a	27/07/88	3	1.37	1.74	4	5	5	7	5	4
VA9 b	27/07/88	3	1.50	1.74	3	5	5	7	5	4
VB1 a	27/07/88	3	1.55	1.78	3	5	5	7	4	4
VB1 b	27/07/88	3	1.62	2.04	3	5	3	7	5	4
VB10 a	09/08/88	2	1.60	1.77	4	5	5	7	5	4
VB10 b	09/08/88	3	1.53	1.58	4	5	5	7	5	4
VB2 a	09/08/88	3	1.43	1.56	3	5	5	7	5	4
VB2 b	27/07/88	3	1.39	2.25	4	5	5	7	4	4
VB4 a	27/07/88	3	1.39	1.67	3	5	5	7	5	4
VB4 b	27/07/88	3	1.37	1.55	3	5	5	7	5	4
VB5 a	27/07/88	3	1.49	1.80	3	5	5	7	5	4
VB5 b	27/07/88	3	1.51	1.51	4	5	5	7	5	4
VB7 a	08/09/88	3	1.44	1.21	2	5	5	7	5	4
VB7 b	08/09/88	3	1.51	1.61	4	5	5	7	5	4
VB8 a	27/07/88	2	1.55	1.65	3	7	7	7	5	4
VB8 b	27/07/88	3	1.63	1.78	2	5	5	7	5	4
VC10 a	27/07/88	3	1.60	1.88	4	5	5	7	5	4
VC10 b	27/07/88	3	1.65	1.81	4	5	5	7	4	4
VC2 a	27/07/88	3	1.44	1.60	4	5	5	7	5	4
VC2 b	27/07/88	3	1.38	1.59	2	5	5	7	4	3
VC3 a	27/07/88	3	1.45	1.47	4	5	5	7	5	4
VC3 b	27/07/88	3	1.45	1.71	4	5	5	7	5	4
VC4 a	08/09/88	3	1.30	1.50	3	5	5	7	5	4
VC4 b	/ /	0	0.00	0.00	0	0	0	0	0	0
VC5 a	25/08/88	3	1.48	1.55	2	5	5	7	5	4
VC5 b	/ /	0	0.00	0.00	0	0	0	0	0	0
VC6 a	08/09/88	3	1.51	1.80	2	3	2	7	5	4
VC6 b	/ /	0	0.00	0.00	0	0	0	0	0	0
VC7 a	25/08/88	3	1.44	1.49	2	5	5	7	5	4
VC7 b	/ /	0	0.00	0.00	0	0	0	0	0	0
VD1 a	25/08/88	3	1.50	1.55	4	5	5	7	5	4
VD1 b	15/08/88	3	1.47	1.79	3	5	5	7	5	4
VD2 a	08/09/88	3	1.60	1.50	3	5	5	7	5	4
VD2 b	21/09/88	3	1.44	1.79	3	5	5	7	5	4
VD5 a	27/07/88	3	1.57	1.70	2	5	5	7	5	4

Appendix IV
Table 1. Leaf-stem characters

Grouped by plant type			1	2	3	4	5a	5b	5c	6	7
VD5 b	27/07/88		3	1.49	1.62	4	5	5	7	4	4
VD6 a	27/07/88		3	1.33	1.39	4	5	5	7	5	4
VD6 b	27/07/88		3	1.47	1.62	3	5	5	7	4	4
VD7 a	27/07/88		3	1.47	1.76	4	5	5	7	5	4
VD7 b	27/07/88		3	1.61	1.62	4	5	5	7	5	4
VE1 a	27/07/88		3	1.62	1.56	3	7	5	7	5	4
VE1 b	27/07/88		3	1.71	1.76	4	5	5	7	5	4
VE10 a	/ /		0	0.00	0.00	0	0	0	0	0	0
VE10 b	/ /		0	0.00	0.00	0	0	0	0	0	0
VE3 a	27/07/88		3	1.50	1.59	4	5	5	7	5	4
VE3 b	27/07/88		3	1.51	1.56	3	5	5	7	4	4
VE4 a	/ /		0	0.00	0.00	0	0	0	0	0	0
VE4 b	/ /		0	0.00	0.00	0	0	0	0	0	0
VE5 a	/ /		0	0.00	0.00	0	0	0	0	0	0
VE5 b	25/08/88		3	1.49	1.69	2	5	5	7	5	4
VE6 a	/ /		0	0.00	0.00	0	0	0	0	0	0
VE6 b	08/09/88		3	1.58	1.63	4	5	5	7	5	4
VE8 a	27/07/88		3	1.43	1.58	4	5	5	7	5	3
VE8 b	27/07/88		3	1.43	1.79	3	5	5	7	5	4
VE9 b	08/09/88		3	1.42	1.74	3	5	5	7	5	4
VH1 a	09/08/88		3	1.53	1.57	3	5	5	7	5	4
VH1 b	09/08/88		3	1.27	1.60	4	5	5	7	5	4
VH10 a	15/08/88		2	1.45	1.82	3	7	5	7	5	4
VH10 b	15/08/88		3	1.23	1.55	3	5	5	7	5	4
VH2 a	27/07/88		3	1.63	2.39	4	5	5	7	5	4
VH2 b	27/07/88		3	1.45	1.57	3	5	5	7	5	4
VH3 a	/ /		0	0.00	0.00	0	0	0	0	0	0
VH3 b	08/09/88		3	1.52	1.62	3	5	5	7	5	4
VH7 a	27/07/88		3	1.57	1.68	3	5	5	7	5	4
VH7 b	27/07/88		3	1.63	1.70	3	7	5	7	5	4
VH8 a	27/07/88		3	1.60	1.77	3	5	5	7	5	4
VH8 b	27/07/88		3	1.66	1.45	3	5	5	7	4	4
VH9 a	27/07/88		3	1.54	1.50	4	5	5	7	5	4
VH9 b	15/08/88		2	1.30	1.70	4	7	5	7	5	4
VX1 a	09/08/88		3	1.17	2.04	3	5	5	7	5	4
VX1 b	09/08/88		3	1.41	1.65	3	5	5	7	5	4
VX2 a	/ /		0	0.00	0.00	0	0	0	0	0	0
VX2 b	25/08/88		3	1.60	1.72	3	5	5	7	5	4
VX3 a	/ /		0	0.00	0.00	0	0	0	0	0	0
VX3 b	25/08/88		3	1.51	1.40	2	5	5	7	5	4
VX4 a	21/09/88		3	1.39	1.60	3	5	5	7	5	4
VX4 b	/ /		0	0.00	0.00	0	0	0	0	0	0

Appendix IV
Table 2. Flower & tuber characters

Grouped by plant type			1	2	3	4	5	6	7	8
** Plant type = CON										
A a	/ /		0	0	0	0	0	5	4	0
A b	/ /		0	0	0	0	0	5	4	4
A c	/ /		0	0	0	0	0	5	4	4
A d	/ /		0	0	0	0	0	0	0	0
B a	/ /		0	0	0	0	0	0	4	0
B b	/ /		0	0	0	0	0	5	4	0
B c	/ /		0	0	0	0	0	0	0	0
B d	/ /		0	0	0	0	0	5	4	0
C a	25/08/88		3	2	1	2	1	5	4	4
C b	/ /		0	0	0	0	0	5	4	4
C c	09/08/88		3	2	1	2	2	5	4	4
C d	08/09/88		3	2	1	2	1	5	4	4
D a	25/08/88		3	2	1	2	1	5	4	4
D b	09/08/88		3	2	1	2	1	5	4	4
D c	25/08/88		3	2	1	2	1	5	4	4
D d	/ /		0	0	0	0	0	5	4	4
E a	25/08/88		3	2	1	2	1	5	4	4
E b	21/09/88		3	2	1	2	1	5	4	0
E c	25/08/88		3	2	1	2	1	5	4	4
E d	/ /		0	0	0	0	0	5	4	4
H a	09/08/88		3	2	1	2	1	5	4	4
H b	25/08/88		3	2	1	2	1	5	4	0
H c	/ /		0	0	0	0	0	0	0	0
H d	09/08/88		3	2	1	2	1	5	4	4

Appendix IV
Table 2. Flower & tuber characters

Grouped by plant type

		1	2	3	4	5	6	7	8
** Plant type = MP									
A2111.1	08/09/88	3	2	1	2	1	5	4	4
A2111.2	/ /	0	0	0	0	0	0	0	0
A2113.1	08/09/88	3	2	1	2	1	5	4	4
A2113.2	/ /	0	0	0	0	0	5	4	0
A2123.1	/ /	0	0	0	0	0	5	4	4
A2511.1	/ /	0	0	0	0	0	0	0	0
A2511.2	21/09/88	3	2	1	2	1	5	4	0
A2512.1	/ /	0	0	0	0	0	0	4	0
A2512.2	21/09/88	3	2	1	2	1	5	4	4
A2521.1	08/09/88	3	2	1	2	1	5	4	0
A63111.1	/ /	0	0	0	0	0	0	0	0
A63111.2	27/07/88	3	2	1	3	1	5	4	4
A63112.1	21/09/88	3	2	1	2	1	5	4	0
A63112.3	08/09/88	3	2	1	2	1	5	4	4
A63312.1	/ /	0	0	0	0	0	0	4	0
A63321.1	27/07/88	3	2	1	3	2	5	4	4
A63321.2	08/09/88	3	2	1	2	1	0	4	0
A63322.2	27/07/88	3	2	1	3	2	5	4	4
A63322.3	15/08/88	3	2	1	2	2	5	4	4
B1211.1	09/08/88	3	2	1	2	2	5	4	4
B1211.2	/ /	0	0	0	0	0	0	0	0
B1213.1	27/07/88	3	2	1	3	2	5	4	4
B1221.2	21/09/88	3	2	1	2	2	5	4	4
B1222.1	08/09/88	3	2	1	2	1	5	4	4
B1222.2	27/07/88	3	2	1	3	2	5	4	4
B1522.2	/ /	0	0	0	0	0	0	4	0
B15231.1	27/07/88	3	2	1	3	2	5	4	4
B15231.2	/ /	0	0	0	0	0	0	0	0
B1531.1	21/09/88	3	2	1	2	1	5	4	4
B1531.2	08/09/88	3	2	1	2	1	5	4	4
B1533.1	09/08/88	3	2	1	2	2	5	4	4
B1533.2	21/09/88	3	2	1	2	1	5	4	0
B4222.1	21/09/88	3	2	1	2	1	5	4	4
B4222.2	27/07/88	3	2	1	3	2	5	4	4
B4231.1	08/09/88	3	2	1	2	2	3	4	0
B4231.2	09/08/88	3	2	1	2	2	5	4	4
B42321.1	08/09/88	3	2	1	2	2	5	4	4
B42321.2	08/09/88	3	2	1	2	2	5	4	0
B4413.2	21/09/88	3	2	1	2	1	5	4	0
B4431.1	/ /	0	0	0	0	0	0	4	0
B4432.2	27/07/88	3	2	1	3	2	5	4	4
C3312.1	09/08/88	3	2	1	2	2	5	4	4
C3312.2	27/07/88	3	2	1	3	2	5	4	4
C3313.1	/ /	0	0	0	0	0	0	0	0
C3313.2	27/07/88	3	2	1	3	2	5	4	4
C3332.1	27/07/88	3	2	1	3	2	5	4	4
C3332.2	27/07/88	3	2	1	3	2	5	4	4

Appendix IV
Table 2. Flower & tuber characters

		Grouped by plant type							
		1	2	3	4	5	6	7	8
C3333.1	27/07/88	3	2	1	3	2	5	4	4
C3333.2	27/07/88	3	2	1	3	2	5	4	4
C3511.2	08/09/88	3	2	1	2	1	5	4	0
C3513.2	21/09/88	3	2	1	2	1	0	4	0
C3521.1	21/09/88	3	2	1	2	1	5	4	4
C3521.2	25/08/88	3	2	1	2	2	5	4	4
C3522.1	/ /	0	0	0	0	0	0	0	0
C6411.2	08/09/88	3	2	1	2	2	5	4	0
C6413.2	09/08/88	3	2	1	2	1	5	4	4
C6432.1	/ /	0	0	0	0	0	0	4	4
C6432.2	/ /	0	0	0	0	0	0	0	0
C6512.1	/ /	0	0	0	0	0	0	0	0
C6512.2	27/07/88	3	2	1	3	2	5	4	4
C6513.1	08/09/88	3	2	1	3	2	5	4	0
C6513.2	/ /	0	0	0	0	0	0	4	0
C6521.1	/ /	0	0	0	0	0	5	4	4
C6521.2	08/09/88	3	2	1	3	2	5	4	4
D3111.2	08/09/88	3	2	1	2	2	5	4	0
D3113.2	09/08/88	3	2	1	2	2	5	4	4
D3121.2	/ /	0	0	0	0	0	0	4	0
D3123.1	/ /	0	0	0	0	0	0	0	0
D3123.2	/ /	0	0	0	0	0	0	0	0
D3512.1	27/07/88	3	2	1	3	2	5	4	4
D3512.2	08/09/88	3	2	1	2	2	5	4	0
D3521.2	21/09/88	3	2	1	2	1	5	4	0
D3523.1	21/09/88	3	2	1	2	2	5	4	0
D3523.2	27/07/88	3	2	1	3	2	5	4	4
D5221.2	21/09/88	3	2	1	2	1	5	4	0
D5223.1	15/08/88	3	2	1	2	2	5	4	4
D52241.1	/ /	0	0	0	0	0	0	4	0
D52241.2	08/09/88	3	2	1	2	1	5	4	4
D52242.1	27/07/88	3	2	1	3	2	5	4	4
D52242.2	27/07/88	3	2	1	3	2	2	4	4
D5412.1	/ /	0	0	0	0	0	5	4	0
D5412.2	/ /	0	0	0	0	0	5	4	4
D5413.1	21/09/88	3	2	1	2	2	5	4	4
D5432.1	/ /	0	0	0	0	0	0	0	0
D5432.2	27/07/88	3	2	1	3	2	5	4	4
D5433.2	/ /	0	0	0	0	0	0	0	0
E1122.1	/ /	0	0	0	0	0	0	0	0
E1123.1	21/09/88	3	2	1	2	1	5	4	0
E1123.2	27/07/88	3	2	1	3	2	5	4	4
E1131.1	08/09/88	3	2	1	2	2	5	4	0
E1132.1	/ /	0	0	0	0	0	5	4	0
E1522.1	/ /	0	0	0	0	0	0	4	0
E1533.1	27/07/88	3	2	1	3	2	5	4	4
E1533.2	15/08/88	3	2	1	2	2	5	4	4
E5223.1	/ /	0	0	0	0	0	5	4	0

Appendix IV
Table 2. Flower & tuber characters

		Grouped by plant type							
		1	2	3	4	5	6	7	8
E5223.2	27/07/88	3	2	1	3	2	5	4	4
E5232.1	08/09/88	3	2	1	2	2	5	4	4
E5232.2	21/09/88	3	2	1	2	1	0	4	0
E5233.1	/ /	0	0	0	0	0	0	0	0
E5311.1	21/09/88	3	2	1	2	1	5	4	4
E5311.2	27/07/88	3	2	1	3	2	5	4	4
E5312.1	21/09/88	3	2	1	2	1	5	4	4
E5312.2	21/09/88	3	2	1	2	1	0	4	0
E5322.1	27/07/88	3	2	1	3	2	5	4	0
E5322.2	15/08/88	3	2	1	2	2	5	4	4
E5323.1	08/09/88	3	2	1	2	2	5	4	4
E5323.2	27/07/88	3	2	1	3	2	5	4	4
H1113.2	25/08/88	3	2	1	2	1	0	4	0
H1511.1	09/08/88	3	2	1	2	2	5	4	4
H1511.2	08/09/88	3	2	1	2	2	5	4	4
H1532.1	/ /	0	0	0	0	0	0	0	0
H1533.1	/ /	0	0	0	0	0	0	0	0
H1533.2	27/07/88	3	2	1	3	2	5	4	4
H2212.2	08/09/88	3	2	1	2	1	0	4	4
H2222.1	08/09/88	3	2	1	3	2	5	4	4
H2222.2	25/08/88	3	2	1	2	2	5	4	4
H2223.2	08/09/88	3	2	1	3	2	3	4	0
H2411.1	08/09/88	0	0	0	0	1	5	4	0
H2413.1	08/09/88	3	2	1	3	2	5	4	0
H2413.2	08/09/88	3	2	1	2	2	5	4	0
H2431.1	27/07/88	3	2	1	3	2	5	4	4
H2431.2	/ /	0	0	0	0	0	5	4	0
H2432.1	27/07/88	3	2	1	3	2	5	4	4
H2432.2	/ /	0	0	0	0	0	0	0	0
H2511.2	27/07/88	3	2	1	3	2	5	4	4
H2513.1	08/09/88	3	2	1	2	2	5	4	4
H2513.2	/ /	0	0	0	0	0	5	4	0
H2521.1	15/08/88	3	2	1	2	1	5	4	4
H2523.1	/ /	0	0	0	0	0	0	0	0
H2523.2	27/07/88	3	2	1	3	2	5	4	4

Appendix IV
Table 2. Flower & tuber characters

Grouped by plant type			1	2	3	4	5	6	7	8
** Plant type = CST										
A.L12 a	/	/	0	0	0	0	0	0	0	0
A.L12 b	/	/	0	0	0	0	0	0	0	0
B.D6 a	/	/	0	0	0	0	0	0	0	0
B.D6 b	/	/	0	0	0	0	0	5	4	4
B.L12 a	/	/	0	0	0	0	0	5	4	4
B.L12 b	/	/	0	0	0	0	0	5	4	0
C.D6 a	/	/	0	0	0	0	0	0	0	0
C.D6 b	/	/	0	0	0	0	0	0	0	0
C.L12 a	25/08/88		3	2	1	2	1	5	4	4
C.L12 b	08/09/88		3	2	1	2	2	5	4	4
C.L6 a	/	/	0	0	0	0	0	0	0	0
C.L6 b	/	/	0	0	0	0	0	5	4	4
D.D6 a	/	/	0	0	0	0	0	0	0	0
D.D6 b	/	/	0	0	0	0	0	5	4	4
D.L12 a	25/08/88		3	2	1	2	1	5	4	4
D.L12 b	/	/	0	0	0	0	0	5	4	0
D.L6 a	/	/	0	0	0	0	0	5	4	4
D.L6 b	08/09/88		3	2	1	2	1	5	4	4
E.D6 a	25/08/88		3	2	1	2	1	5	4	4
E.D6 b	/	/	0	0	0	0	0	5	4	0
E.L12 a	/	/	0	0	0	0	0	0	4	4
E.L12 b	/	/	0	0	0	0	0	5	4	4
E.L6 a	/	/	0	0	0	0	0	0	0	0
E.L6 b	/	/	0	0	0	0	0	0	4	0
H.D6 a	/	/	0	0	0	0	0	5	4	4
H.D6 b	15/08/88		3	2	1	2	1	5	4	4
H.L12 a	08/09/88		3	2	1	2	1	5	4	4
H.L12 b	/	/	0	0	0	0	0	5	4	4
H.L6 a	/	/	0	0	0	0	0	0	0	0
H.L6 b	/	/	0	0	0	0	0	5	4	0

Appendix IV
Table 2. Flower & tuber characters

Grouped by plant type

		1	2	3	4	5	6	7	8
** Plant type = OST									
SA1 b	/ /	0	0	0	0	0	0	0	0
SA2 a	/ /	0	0	0	0	0	0	0	0
SA2 b	/ /	0	0	0	0	0	0	4	0
SA4 a	21/09/88	3	2	1	2	1	0	4	0
SA4 b	08/09/88	3	2	1	2	1	5	4	4
SB1 a	/ /	0	0	0	0	0	0	0	0
SB1 b	08/09/88	3	2	1	2	1	5	4	4
SB2 a	08/09/88	3	2	1	2	1	5	4	4
SB2 b	08/09/88	3	2	1	2	2	5	4	0
SB4 a	21/09/88	3	2	1	2	1	0	4	0
SB4 b	/ /	0	0	0	0	0	0	0	0
SC2 a	08/09/88	3	2	1	2	1	5	4	4
SC2 b	/ /	0	0	0	0	0	0	0	0
SD1 a	21/09/88	3	2	1	2	1	0	4	4
SD1 b	08/09/88	3	2	1	2	1	5	4	4
SD2 a	15/08/88	3	2	1	2	1	5	4	4
SD2 b	/ /	0	0	0	0	0	0	0	0
SD4 a	/ /	0	0	0	0	0	0	0	0
SD4 b	/ /	0	0	0	0	0	5	4	0
SE1 a	/ /	0	0	0	0	0	0	4	0
SE1 b	09/08/88	3	2	1	2	1	5	4	4
SE2 a	25/08/88	3	2	1	3	1	5	4	4
SE2 b	15/08/88	3	2	1	2	1	5	4	4
SE3 a	21/09/88	3	2	1	2	1	5	4	4
SE3 b	15/08/88	3	2	1	2	1	5	4	4
SH1 a	/ /	0	0	0	0	0	0	0	0
SH2 a	/ /	0	0	0	0	0	5	4	0
SH2 b	15/08/88	3	2	1	2	1	5	4	4
SH3 a	09/08/88	3	2	1	2	2	5	4	4
SH3 b	/ /	0	0	0	0	0	0	0	0
SH4 a	/ /	0	0	0	0	0	5	4	4
SH4 b	/ /	0	0	0	0	0	5	4	0

Appendix IV
Table 2. Flower & tuber characters

		Grouped by plant type							
		1	2	3	4	5	6	7	8
** Plant type = REG									
VA1 a	09/08/88	3	2	2	2	1	5	4	4
VA1 b	27/07/88	3	2	1	2	2	5	4	4
VA10 a	08/09/88	3	2	2	2	2	5	4	4
VA10 b	/ /	0	0	0	0	0	5	4	0
VA3 a	15/08/88	3	2	1	2	1	5	4	4
VA3 b	/ /	0	0	0	0	0	3	4	4
VA4 a	/ /	0	0	0	0	0	0	4	0
VA4 b	/ /	0	0	0	0	0	0	0	0
VA5 a	27/07/88	4	2	1	2	1	5	4	4
VA5 b	09/08/88	4	1	2	3	1	5	4	2
VA8 a	/ /	0	0	0	0	0	0	4	0
VA8 b	08/09/88	3	2	1	2	1	5	4	4
VA9 a	27/07/88	3	2	1	2	1	5	4	4
VA9 b	27/07/88	3	2	1	3	2	5	4	4
VB1 a	27/07/88	3	2	1	3	2	5	4	4
VB1 b	27/07/88	0	0	0	0	1	3	4	4
VB10 a	09/08/88	3	2	1	2	1	5	4	4
VB10 b	09/08/88	3	2	1	2	2	5	4	4
VB2 a	09/08/88	3	2	1	2	1	5	4	4
VB2 b	27/07/88	3	2	1	3	2	5	4	4
VB4 a	27/07/88	3	2	1	3	2	5	4	4
VB4 b	27/07/88	3	2	1	3	2	5	4	4
VB5 a	27/07/88	3	2	1	3	2	5	4	4
VB5 b	27/07/88	3	2	1	3	2	5	4	4
VB7 a	08/09/88	3	2	1	2	1	0	4	4
VB7 b	08/09/88	3	2	1	2	1	5	4	0
VB8 a	27/07/88	2	2	1	3	2	5	4	4
VB8 b	27/07/88	3	2	1	3	2	5	4	4
VC10 a	27/07/88	3	2	1	3	2	5	4	4
VC10 b	27/07/88	3	2	1	3	2	5	4	4
VC2 a	27/07/88	3	2	1	3	2	5	4	4
VC2 b	27/07/88	3	2	1	3	2	5	4	4
VC3 a	27/07/88	3	2	1	3	2	5	4	4
VC3 b	27/07/88	3	2	1	3	2	5	4	4
VC4 a	08/09/88	3	2	1	2	1	5	4	4
VC4 b	/ /	0	0	0	0	0	0	4	0
VC5 a	25/08/88	3	2	1	2	1	5	4	0
VC5 b	/ /	0	0	0	0	0	0	4	0
VC6 a	08/09/88	3	2	1	2	1	0	2	2
VC6 b	/ /	0	0	0	0	0	5	4	4
VC7 a	25/08/88	3	2	1	3	1	5	4	0
VC7 b	/ /	0	0	0	0	0	0	0	0
VD1 a	25/08/88	3	2	1	2	2	5	4	4
VD1 b	15/08/88	3	2	1	2	2	5	4	4
VD2 a	08/09/88	3	2	1	2	1	5	4	4
VD2 b	21/09/88	3	2	1	2	1	5	4	4
VD5 a	27/07/88	3	2	1	3	1	5	4	4

Appendix IV
Table 2. Flower & tuber characters

Grouped by plant type			1	2	3	4	5	6	7	8
VD5 b	27/07/88		3	2	1	2	2	5	4	4
VD6 a	27/07/88		3	2	1	2	2	5	4	4
VD6 b	27/07/88		3	2	1	3	1	3	4	4
VD7 a	27/07/88		3	2	1	3	2	5	4	4
VD7 b	27/07/88		3	2	1	2	2	5	4	4
VE1 a	27/07/88		3	2	1	3	2	0	0	0
VE1 b	27/07/88		3	2	1	3	1	5	4	4
VE10 a	/ /		0	0	0	0	0	0	0	0
VE10 b	/ /		0	0	0	0	0	0	0	0
VE3 a	27/07/88		3	2	1	2	2	5	4	4
VE3 b	27/07/88		3	2	1	3	2	5	4	4
VE4 a	/ /		0	0	0	0	0	0	0	0
VE4 b	/ /		0	0	0	0	0	0	4	0
VE5 a	/ /		0	0	0	0	0	0	4	0
VE5 b	25/08/88		3	2	1	2	1	5	4	4
VE6 a	/ /		0	0	0	0	0	0	4	0
VE6 b	08/09/88		3	2	2	2	1	5	4	0
VE8 a	27/07/88		3	2	1	3	2	5	4	4
VE8 b	27/07/88		3	2	1	3	1	5	4	4
VE9 b	08/09/88		3	2	1	2	1	5	4	4
VH1 a	09/08/88		3	2	1	2	1	5	4	4
VH1 b	09/08/88		3	2	1	2	1	5	4	4
VH10 a	15/08/88		3	2	2	1	1	5	4	4
VH10 b	15/08/88		3	2	1	2	2	5	4	4
VH2 a	27/07/88		3	2	1	3	2	5	4	4
VH2 b	27/07/88		3	2	1	3	2	5	4	4
VH3 a	/ /		0	0	0	0	0	0	0	0
VH3 b	08/09/88		3	2	1	2	2	5	4	4
VH7 a	27/07/88		3	2	1	3	2	5	4	4
VH7 b	27/07/88		3	2	1	3	2	5	4	4
VH8 a	27/07/88		3	2	1	3	2	5	4	4
VH8 b	27/07/88		3	2	1	3	2	5	4	4
VH9 a	27/07/88		2	2	1	3	1	5	4	4
VH9 b	15/08/88		3	2	2	2	1	5	4	4
VX1 a	09/08/88		3	2	1	2	2	5	4	4
VX1 b	09/08/88		3	2	1	2	2	5	4	4
VX2 a	/ /		0	0	0	0	0	5	0	0
VX2 b	25/08/88		3	2	1	2	1	5	4	4
VX3 a	/ /		0	0	0	0	0	0	4	0
VX3 b	25/08/88		3	2	1	2	1	5	4	4
VX4 a	21/09/88		3	2	1	2	1	5	4	0
VX4 b	/ /		0	0	0	0	0	5	2	0

Appendix IV
Table 3. Tuber numbers & weights

Grouped by plant type											
		1		2		3		4		5	6
** Plant type = CON											
A a	1	9.0	3	68.6	1	31.7	0	0.0	0	0.0	0.0
A b	2	10.8	0	0.0	3	146.2	1	197.7	0	0.0	0.0
A c	4	8.9	1	10.2	0	0.0	0	0.0	2	454.9	0.0
A d	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0.0
B a	0	0.0	0	0.0	1	46.7	0	0.0	0	0.0	0.0
B b	3	10.3	1	22.5	1	53.6	1	109.6	0	0.0	0.0
B c	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0.0
B d	0	0.0	0	0.0	2	70.7	1	171.5	0	0.0	0.0
C a	3	3.1	0	0.0	1	36.8	1	102.8	1	230.3	0.0
C b	3	6.0	3	61.4	4	230.8	3	450.0	0	0.0	0.0
C c	10	32.2	7	111.2	10	578.3	7	1055.7	4	1220.5	0.0
C d	2	11.5	2	39.1	3	165.3	3	474.5	0	0.0	0.0
D a	26	124.5	14	242.9	11	643.2	4	547.8	1	262.9	0.0
D b	2	10.7	4	81.3	0	0.0	1	169.7	2	603.1	0.0
D c	2	6.9	2	41.1	1	34.9	2	303.8	1	377.7	0.0
D d	12	40.1	3	52.3	5	213.6	0	0.0	1	207.6	0.0
E a	4	3.4	6	120.1	4	212.0	2	220.1	1	263.3	0.0
E b	0	0.0	1	11.5	2	171.8	1	157.8	0	0.0	0.0
E c	11	67.7	3	42.3	4	212.5	3	455.0	0	0.0	0.0
E d	10	34.3	6	101.7	3	194.5	2	277.8	0	0.0	0.0
H a	3	9.0	0	0.0	1	63.2	4	689.2	1	340.1	0.0
H b	5	17.7	4	94.6	3	221.2	1	119.6	1	241.0	0.0
H c	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0.0
H d	5	28.7	1	19.9	6	421.8	3	350.3	1	248.7	0.0

Appendix IV
Table 3. Tuber numbers & weights

Grouped by plant type

	1	2	3	4	5	6
** Plant type = MP						
A2111.1	7	30.9	2	36.6	3	185.1
A2111.2	0	0.0	0	0.0	0	0.0
A2113.1	2	9.5	6	129.3	21	17.5
A2113.2	1	6.6	1	25.4	2	109.3
A2123.1	1	9.3	2	24.9	4	184.2
A2511.1	3	18.4	0	0.0	0	0.0
A2511.2	1	8.0	5	114.2	2	135.8
A2512.1	1	2.4	1	10.3	1	30.1
A2512.2	3	19.4	0	0.0	2	99.4
A2521.1	0	0.0	0	0.0	3	200.6
A63111.1	2	1.9	0	0.0	0	0.0
A63111.2	13	54.4	6	82.6	10	632.1
A63112.1	10	38.9	7	126.8	4	163.2
A63112.3	3	12.6	2	40.9	3	193.0
A63312.1	0	0.0	1	27.5	1	54.4
A63321.1	24	93.6	16	285.2	7	385.5
A63321.2	2	12.0	3	66.6	1	46.1
A63322.2	5	27.0	4	60.9	7	442.0
A63322.3	0	0.0	0	0.0	1	48.0
B1211.1	15	77.8	13	288.1	6	262.8
B1211.2	0	0.0	0	0.0	0	0.0
B1213.1	11	44.3	10	210.1	7	371.1
B1221.2	0	0.0	3	60.8	0	0.0
B1222.1	1	8.7	1	15.9	4	293.3
B1222.2	43	168.7	13	246.6	24	1212.0
B1522.2	1	1.3	0	0.0	1	56.3
B15231.1	65	176.1	17	320.6	19	1019.2
B15231.2	0	0.0	1	10.6	0	0.0
B1531.1	6	16.4	3	54.7	2	77.9
B1531.2	3	22.2	5	82.7	2	136.0
B1533.1	13	54.5	9	178.8	6	377.7
B1533.2	3	13.9	2	23.4	4	174.1
B4222.1	2	4.6	0	0.0	0	0.0
B4222.2	27	106.8	21	354.3	27	1680.0
B4231.1	4	10.0	2	45.3	1	50.1
B4231.2	19	73.0	12	221.1	11	677.1
B42321.1	2	11.9	1	20.6	4	283.3
B42321.2	5	21.1	1	19.9	3	162.6
B4413.2	6	11.6	2	51.8	3	185.8
B4431.1	0	0.0	0	0.0	1	49.5
B4432.2	14	57.5	7	148.8	16	1081.1
C3312.1	8	38.7	11	212.8	17	951.7
C3312.2	40	144.6	21	337.4	6	308.4
C3313.1	0	0.0	1	11.1	1	31.6
C3313.2	25	90.2	18	342.4	9	613.7
C3332.1	18	60.1	4	71.6	7	473.4
C3332.2	25	99.3	7	126.3	3	181.6

Appendix IV
Table 3. Tuber numbers & weights

Grouped by plant type

		1		2		3		4		5		6
C3333.1	17	71.7	9	177.3	9	485.9	3	430.3	6	1499.6	0	0.0
C3333.2	20	87.6	10	215.9	13	719.9	6	927.3	1	202.0	0	0.0
C3511.2	2	17.9	0	0.0	5	261.9	0	0.0	5	550.9	0	0.0
C3513.2	0	0.0	2	31.1	0	0.0	0	0.0	1	267.4	0	0.0
C3521.1	1	4.2	3	44.1	3	136.2	1	115.8	0	0.0	0	0.0
C3521.2	5	16.9	2	35.3	4	193.4	4	510.0	1	417.3	0	0.0
C3522.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
C6411.2	4	18.8	1	18.1	1	66.4	2	263.8	0	0.0	0	0.0
C6413.2	2	8.3	6	98.4	9	479.4	3	549.3	1	305.8	0	0.0
C6432.1	2	6.5	1	13.7	1	42.1	0	0.0	0	0.0	0	0.0
C6432.2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
C6512.1	2	6.0	0	0.0	1	52.6	0	0.0	0	0.0	0	0.0
C6512.2	38	155.9	12	229.8	13	770.3	6	727.3	3	784.0	1	910.1
C6513.1	0	0.0	1	23.4	1	53.7	1	128.9	0	0.0	0	0.0
C6513.2	3	8.7	2	33.3	1	36.8	0	0.0	0	0.0	0	0.0
C6521.1	1	3.5	3	39.3	3	169.8	1	152.5	0	0.0	0	0.0
C6521.2	2	6.4	1	25.3	2	97.2	1	100.7	0	0.0	0	0.0
D3111.2	7	21.5	6	105.6	10	556.8	0	0.0	0	0.0	0	0.0
D3113.2	14	49.3	7	163.0	13	775.1	4	686.8	2	535.8	0	0.0
D3121.2	1	2.5	1	13.4	3	136.6	0	0.0	0	0.0	0	0.0
D3123.1	1	7.6	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
D3123.2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
D3512.1	10	45.0	4	67.8	14	779.6	3	392.1	4	1073.5	0	0.0
D3512.2	4	13.4	1	22.8	0	0.0	1	124.7	0	0.0	0	0.0
D3521.2	0	0.0	3	60.1	0	0.0	1	113.7	0	0.0	0	0.0
D3523.1	0	0.0	2	47.8	1	32.0	1	144.5	0	0.0	0	0.0
D3523.2	20	81.2	16	297.2	14	740.0	4	521.2	2	802.5	1	664.5
D5221.2	1	7.7	2	40.0	1	39.3	1	197.4	0	0.0	0	0.0
D5223.1	14	35.6	10	240.3	10	574.9	5	662.7	0	0.0	0	0.0
D52241.1	1	2.9	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
D52241.2	4	20.4	4	50.2	4	201.9	1	166.9	1	209.1	0	0.0
D52242.1	13	44.2	5	95.0	11	652.4	4	581.4	3	1170.8	0	0.0
D52242.2	5	18.2	7	140.2	8	594.7	5	668.4	4	1618.3	0	0.0
D5412.1	1	9.0	1	17.4	0	0.0	1	172.4	0	0.0	0	0.0
D5412.2	5	21.0	4	73.2	0	0.0	3	516.7	0	0.0	0	0.0
D5413.1	1	2.4	1	11.4	1	55.1	1	192.3	1	238.0	0	0.0
D5432.1	0	0.0	0	0.0	2	79.5	0	0.0	0	0.0	0	0.0
D5432.2	5	28.5	9	173.7	6	352.1	7	934.8	3	1243.2	0	0.0
D5433.2	2	6.7	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
E1122.1	3	6.5	1	28.6	0	0.0	0	0.0	0	0.0	0	0.0
E1123.1	0	0.0	0	0.0	1	76.9	1	112.9	0	0.0	0	0.0
E1123.2	7	38.5	2	34.7	6	436.0	1	142.5	3	924.4	1	515.3
E1131.1	7	26.5	1	26.7	3	155.5	0	0.0	0	0.0	0	0.0
E1132.1	2	2.2	4	60.1	2	108.6	0	0.0	0	0.0	0	0.0
E1522.1	1	1.2	1	27.8	0	0.0	0	0.0	0	0.0	0	0.0
E1533.1	7	34.3	7	138.3	9	611.3	4	573.7	3	695.4	0	0.0
E1533.2	20	68.1	10	181.2	13	725.6	4	462.9	1	382.2	0	0.0
E5223.1	3	8.5	3	61.6	0	0.0	1	122.9	0	0.0	0	0.0

Appendix IV

Table 3. Tuber numbers & weights

Grouped by plant type

	1	2	3	4	5	6
E5223.2	27	84.5	7	126.5	9	509.8
E5232.1	3	10.6	3	58.1	3	178.2
E5232.2	0	0.0	0	0.0	3	170.9
E5233.1	0	0.0	1	21.2	0	0.0
E5311.1	1	7.4	2	45.0	0	0.0
E5311.2	25	111.5	17	349.0	10	527.8
E5312.1	1	2.9	0	0.0	2	127.4
E5312.2	0	0.0	0	0.0	0	0.0
E5322.1	18	81.4	12	221.7	10	561.5
E5322.2	30	126.1	14	284.4	9	512.4
E5323.1	4	22.8	0	0.0	3	228.7
E5323.2	11	51.1	8	126.9	6	333.2
H1113.2	2	9.1	1	16.8	5	154.3
H1511.1	11	47.6	6	139.0	5	306.6
H1511.2	3	17.3	2	25.5	3	126.5
H1532.1	5	30.6	1	17.1	0	0.0
H1533.1	0	0.0	0	0.0	0	0.0
H1533.2	10	34.0	9	156.2	4	242.6
H2212.2	4	13.2	2	32.2	2	84.9
H2222.1	1	5.6	0	0.0	0	0.0
H2222.2	6	31.5	4	63.4	4	212.5
H2223.2	1	7.9	2	30.3	2	131.9
H2411.1	2	12.2	1	11.1	4	267.2
H2413.1	1	3.7	2	42.0	2	129.5
H2413.2	2	14.6	3	40.8	3	159.8
H2431.1	51	118.9	12	213.0	11	544.5
H2431.2	0	0.0	3	62.3	0	0.0
H2432.1	11	50.2	4	70.9	11	702.9
H2432.2	2	6.9	0	0.0	0	0.0
H2511.2	19	45.5	10	172.7	10	526.1
H2513.1	4	11.4	3	46.3	2	142.8
H2513.2	3	8.1	3	48.5	2	105.9
H2521.1	9	39.0	4	91.5	6	309.5
H2523.1	0	0.0	0	0.0	0	0.0
H2523.2	15	50.0	5	120.8	5	234.9

Appendix IV
Table 3. Tuber numbers & weights

Grouped by plant type

	1	2	3	4	5	6
** Plant type = CST						
A.L12 a	0	0.0	0	0.0	0	0.0
A.L12 b	0	0.0	0	0.0	0	0.0
B.D6 a	0	0.0	0	0.0	0	0.0
B.D6 b	1	0.9	4	77.7	8	406.6
B.L12 a	10	41.8	6	130.4	2	140.1
B.L12 b	4	10.6	0	0.0	2	122.4
C.D6 a	0	0.0	0	0.0	0	0.0
C.D6 b	0	0.0	0	0.0	0	0.0
C.L12 a	6	16.9	2	30.7	3	183.0
C.L12 b	16	51.3	4	67.6	6	346.8
C.L6 a	0	0.0	0	0.0	0	0.0
C.L6 b	15	64.0	5	100.0	8	435.0
D.D6 a	9	16.1	1	22.7	5	244.7
D.D6 b	0	0.0	0	0.0	0	0.0
D.L12 a	30	113.0	9	163.2	6	311.4
D.L12 b	13	57.5	4	54.1	8	378.3
D.L6 a	15	25.3	3	48.7	3	135.8
D.L6 b	20	64.7	4	85.8	9	504.3
E.D6 a	7	19.3	4	84.0	7	384.5
E.D6 b	3	10.4	1	10.9	1	45.8
E.L12 a	4	10.8	0	0.0	1	47.2
E.L12 b	5	18.2	3	48.6	1	54.8
E.L6 a	0	0.0	0	0.0	0	0.0
E.L6 b	2	5.0	1	16.7	1	32.4
H.D6 a	14	40.1	4	87.1	5	239.2
H.D6 b	21	90.3	8	147.6	10	599.9
H.L12 a	23	59.7	8	120.7	4	214.4
H.L12 b	4	23.9	8	151.4	2	118.5
H.L6 a	0	0.0	0	0.0	0	0.0
H.L6 b	3	14.0	0	0.0	1	33.9

Appendix IV
Table 3. Tuber numbers & weights

Grouped by plant type											
	1		2		3		4		5		6
** Plant type = OST											
SA1 b	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
SA2 a	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
SA2 b	3	10.0	0	0.0	2	118.1	0	0.0	0	0.0	0
SA4 a	0	0.0	0	0.0	1	40.9	1	114.9	0	0.0	0
SA4 b	2	3.2	2	34.8	3	131.1	2	292.8	1	234.0	0
SB1 a	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
SB1 b	5	31.7	2	36.9	2	169.1	2	311.8	0	0.0	0
SB2 a	4	9.8	5	75.2	5	262.6	1	183.1	0	0.0	0
SB2 b	0	0.0	1	27.8	1	66.4	1	172.1	0	0.0	0
SB4 a	3	3.3	1	24.8	2	90.7	1	123.8	0	0.0	0
SB4 b	1	4.3	0	0.0	0	0.0	0	0.0	0	0.0	0
SC2 a	1	2.5	3	38.2	3	138.4	0	0.0	1	354.8	0
SC2 b	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
SD1 a	0	0.0	0	0.0	2	78.5	1	138.8	0	0.0	0
SD1 b	0	0.0	4	67.0	2	86.7	0	0.0	1	418.0	0
SD2 a	0	0.0	1	13.5	1	89.4	1	113.2	1	251.3	1
SD2 b	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
SD4 a	0	0.0	1	26.1	0	0.0	1	154.1	0	0.0	0
SD4 b	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
SE1 a	3	6.0	3	54.1	0	0.0	0	0.0	0	0.0	0
SE1 b	5	17.2	2	31.6	4	162.7	2	357.9	0	0.0	0
SE2 a	0	0.0	4	73.7	2	125.7	0	0.0	3	1038.0	0
SE2 b	5	30.3	2	31.9	7	460.2	3	346.0	2	619.9	0
SE3 a	7	22.5	1	24.6	4	277.0	1	163.7	0	0.0	0
SE3 b	8	47.5	6	127.7	6	388.2	0	0.0	1	234.4	0
SH1 a	2	9.9	1	12.1	1	31.0	0	0.0	0	0.0	0
SH2 a	0	0.0	0	0.0	1	80.2	1	162.5	1	319.6	0
SH2 b	1	5.4	8	160.4	2	189.4	2	341.6	0	0.0	0
SH3 a	2	1.0	1	22.6	2	109.1	3	413.2	1	398.2	0
SH3 b	3	7.7	1	24.9	0	0.0	0	0.0	0	0.0	0
SH4 a	11	41.3	6	114.5	6	288.7	1	126.0	0	0.0	0
SH4 b	5	22.7	1	10.8	2	100.7	0	0.0	0	0.0	0

Appendix IV
Table 3. Tuber numbers & weights

Grouped by plant type

		1		2		3		4		5		6
** Plant type = REG												
VA1 a	4	8.6	2	28.4	2	125.7	1	103.0	2	473.8	1	571.8
VA1 b	12	42.8	6	86.2	5	317.7	0	0.0	5	1263.7	2	1423.1
VA10 a	0	0.0	3	61.8	0	0.0	1	122.3	0	0.0	1	548.3
VA10 b	0	0.0	0	0.0	0	0.0	1	158.8	0	0.0	0	0.0
VA3 a	7	22.8	2	21.7	3	148.4	2	326.2	1	265.1	0	0.0
VA3 b	1	5.6	0	0.0	1	61.1	4	626.8	2	532.0	0	0.0
VA4 a	1	5.3	0	0.0	1	31.1	1	77.3	0	0.0	0	0.0
VA4 b	1	3.4	1	11.9	1	41.6	0	0.0	0	0.0	0	0.0
VA5 a	0	0.0	3	63.6	1	70.1	2	296.6	1	261.5	1	625.4
VA5 b	9	49.8	7	134.8	4	217.1	2	285.8	2	513.3	0	0.0
VA8 a	0	0.0	1	25.7	0	0.0	0	0.0	0	0.0	0	0.0
VA8 b	4	19.7	1	20.2	1	44.2	2	274.9	0	0.0	0	0.0
VA9 a	0	0.0	3	58.2	1	55.6	3	433.8	2	680.0	1	535.0
VA9 b	6	16.0	2	29.3	5	270.7	2	226.6	1	296.9	1	787.5
VB1 a	4	7.2	2	42.1	3	189.4	4	513.6	2	482.7	0	0.0
VB1 b	0	0.0	5	76.2	3	181.3	1	168.8	2	537.1	1	1148.9
VB10 a	2	2.5	3	47.3	1	70.7	1	171.3	3	830.2	0	0.0
VB10 b	1	5.7	4	71.5	2	93.9	3	387.8	2	653.1	0	0.0
VB2 a	0	0.0	0	0.0	2	69.6	1	105.1	2	893.8	1	5110.3
VB2 b	6	21.7	1	12.4	2	115.1	3	390.1	1	251.6	3	2193.3
VB4 a	0	0.0	1	13.1	2	172.2	4	613.9	1	449.2	1	557.5
VB4 b	1	3.4	1	19.7	10	514.8	1	178.2	3	840.8	2	1401.4
VB5 a	5	17.6	1	25.8	2	74.5	4	512.1	3	742.5	1	763.9
VB5 b	2	3.7	2	41.7	3	136.0	1	100.8	4	1344.7	1	615.8
VB7 a	3	8.2	1	25.9	0	0.0	1	135.7	0	0.0	0	0.0
VB7 b	0	0.0	0	0.0	3	193.9	0	0.0	1	248.2	0	0.0
VB8 a	1	1.1	2	25.2	4	261.2	3	564.8	2	527.4	0	0.0
VB8 b	5	8.7	1	27.8	4	254.6	0	0.0	3	772.6	1	688.4
VC10 a	4	17.7	4	65.5	6	395.6	5	727.2	2	626.8	0	0.0
VC10 b	3	12.8	0	0.0	6	453.5	5	759.0	2	716.8	1	917.0
VC2 a	6	28.9	5	118.3	9	507.1	5	635.9	0	3820.7	0	0.0
VC2 b	5	21.8	1	29.8	1	91.8	2	282.5	2	830.3	0	0.0
VC3 a	1	5.3	2	46.4	4	304.3	3	384.6	2	768.1	0	0.0
VC3 b	2	5.2	2	31.3	1	47.3	1	145.5	4	1503.1	1	564.4
VC4 a	3	8.1	2	38.9	1	49.3	1	139.7	0	0.0	0	0.0
VC4 b	0	0.0	0	0.0	1	53.4	0	0.0	0	0.0	0	0.0
VC5 a	3	7.6	3	48.5	0	0.0	0	0.0	0	0.0	1	754.0
VC5 b	2	17.4	1	21.6	0	0.0	0	0.0	0	0.0	0	0.0
VC6 a	1	5.5	1	22.6	1	30.8	0	0.0	1	281.6	0	0.0
VC6 b	5	15.9	3	56.2	2	109.9	5	760.1	1	481.1	1	539.4
VC7 a	1	4.1	2	23.9	3	280.4	0	0.0	0	0.0	0	0.0
VC7 b	0	0.0	0	0.0	1	53.5	0	0.0	0	0.0	0	0.0
VD1 a	3	5.3	3	47.7	2	109.3	1	155.3	1	204.1	0	0.0
VD1 b	3	20.3	1	13.4	2	138.6	2	211.6	2	702.8	0	0.0
VD2 a	3	15.0	3	51.9	2	137.9	2	260.3	0	0.0	0	0.0
VD2 b	1	3.5	2	43.9	1	31.3	2	295.3	0	0.0	0	0.0
VD5 a	1	6.3	6	81.5	6	238.9	3	465.1	4	909.2	1	663.4

Appendix IV
Table 3. Tuber numbers & weights

Grouped by plant type												
		1		2		3		4		5		6
VD5 b	1	4.1	1	26.5	5	259.1	2	149.9	1	357.6	2	1470.3
VD6 a	0	0.0	1	14.0	2	143.8	3	387.3	0	0.0	*	89.2
VD6 b	4	17.4	4	62.1	5	315.8	3	389.2	3	1091.0	0	0.0
VD7 a	6	26.6	1	25.5	7	433.2	3	429.9	5	1210.2	0	0.0
VD7 b	1	8.7	0	0.0	3	202.8	2	286.8	3	983.4	1	884.5
VE1 a	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
VE1 b	8	44.4	12	236.1	12	745.0	3	367.8	2	489.2	1	741.7
VE10 a	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
VE10 b	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
VE3 a	8	27.1	3	46.5	0	0.0	2	390.9	4	1378.1	1	581.9
VE3 b	6	27.5	1	15.1	4	174.0	8	1092.5	4	1271.5	0	0.0
VE4 a	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
VE4 b	2	14.4	0	0.0	1	74.4	0	0.0	0	0.0	0	0.0
VE5 a	3	14.4	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
VE5 b	6	23.9	4	61.2	3	199.7	0	0.0	3	990.6	0	0.0
VE6 a	0	0.0	0	0.0	0	0.0	1	111.7	0	0.0	0	0.0
VE6 b	0	0.0	2	22.0	4	278.1	1	114.5	0	0.0	0	0.0
VE8 a	11	37.8	3	60.7	5	245.1	3	389.1	3	1181.5	0	0.0
VE8 b	8	31.2	4	100.6	4	271.5	2	315.6	5	1468.6	0	0.0
VE9 b	2	4.9	2	44.8	3	196.4	0	0.0	1	204.2	0	0.0
VH1 a	1	2.1	1	11.4	0	0.0	1	129.5	3	727.1	0	0.0
VH1 b	0	0.0	1	11.1	0	0.0	1	162.6	2	917.2	0	0.0
VH10 a	8	17.4	1	16.8	2	120.8	3	453.7	1	389.0	0	0.0
VH10 b	5	16.3	2	24.2	2	141.6	2	311.0	3	693.2	1	546.5
VH2 a	1	8.2	0	0.0	2	140.1	4	580.0	1	314.0	1	812.9
VH2 b	1	2.0	1	11.6	6	395.7	2	314.8	1	295.6	1	781.1
VH3 a	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
VH3 b	5	19.9	1	18.5	2	102.8	0	0.0	1	223.6	0	0.0
VH7 a	2	4.7	1	14.4	0	0.0	2	365.0	2	788.5	1	1271.1
VH7 b	15	48.3	3	49.4	3	203.2	2	365.5	2	864.3	0	0.0
VH8 a	1	5.0	1	14.6	1	47.1	1	150.8	4	1548.7	1	548.9
VH8 b	1	3.0	1	27.4	7	360.6	2	138.8	5	1766.9	0	0.0
VH9 a	1	1.7	5	81.1	3	159.1	3	352.7	2	887.1	0	0.0
VH9 b	2	5.9	1	27.4	1	41.0	1	137.9	1	305.4	1	584.7
VX1 a	4	3.3	2	39.3	6	259.6	2	374.9	0	0.0	0	0.0
VX1 b	8	49.7	3	61.8	0	0.0	1	123.6	2	622.5	0	0.0
VX2 a	1	9.2	4	68.7	0	0.0	0	0.0	0	0.0	0	0.0
VX2 b	6	20.0	5	77.6	3	219.8	2	348.0	1	255.2	0	0.0
VX3 a	0	0.0	1	27.3	0	0.0	0	0.0	0	0.0	0	0.0
VX3 b	1	6.0	0	0.0	0	0.0	1	147.7	1	240.3	0	0.0
VX4 a	0	0.0	1	11.2	1	36.6	1	146.0	0	0.0	0	0.0
VX4 b	1	1.8	1	13.7	2	151.3	0	0.0	0	0.0	0	0.0

ERRATA

- Page 36: Heading 2.4.5 should read;
Storage of shoot cultures under modified osmotic conditions.
- Page 51: It should be noted that not all plants regenerated from such small meristematic explants are free of virus contamination.

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