Transposon tagging: Towards the isolation of the resistance *R*-genes in potato against *Phytophthora infestans* (Mont.) de Bary

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Bibliographic Abstract: The thesis describes the first step of the research leading to transposon tagging of the resistance genes against late blight of potato (*Phytophthora infestans*). Different diploid potato populations have been developed in which four *R*-genes have been localized on the RFLP map. Screening for high regeneration and transformation competence in a segregating population showed positive correlation between the *R1* gene and the competence for transformation. It resulted also in the selection of a diploid potato clone suitable for transposon tagging. The transformation of this clone with *Ds*-carrying T-DNA and the localization of 60 different T-DNA insertions were carried out. The involvement of other genetic factors in the expression of the *R*-genes was detected.

Statements (stellingen)

1) A carefully planned strategy and strenuous work are keys to success in transposon tagging for isolating plant genes, but the main key probably is good luck (This thesis).

2) Competence for genetic transformation existed in our potato material long before the recent techniques were developed. This suggests lack of selection pressure (or evolution) on this competence (This thesis).

3) Resistant plants can be obtained from two susceptible parents although susceptibility is recessive (This thesis).

4) The presence in a population of an unknown suppressor or enhancer for a certain trait may greatly complicate mapping of this trait (This thesis).

5) It is wiser to tolerate a certain level of late blight attack than to eradicate the fungus.

6) Cloning of genes for race-specific resistance to *Phytophthora infestans* (*R*-genes) does not necessarily aim at controlling this disease, but may be a step in that direction.

7) Theory and practice of breeding make people aware of the shortness of human life.

8) Without students a professor would not be a professor.

9) With all the economic, social and political crises, some people are still willing to give and keep their words.

10) Tell your children the truth about Sinterklaas, they will say: "you are a liar".

Stellingen behorende bij proefschrift getiteld " **Transposon tagging: Towards the isolation of the resistance *R*-genes in potato against *Phytophthora infestans* (Mont.) de Bary**" door Ali El Kharbotly.

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Contents

Chapter 1

Out line of the thesis

The history of the fungus *Phytophthora infestans* begins in the 1840s. It first appeared in 1843 in the U.S.A and two years later in Europe. A well documented report recorded that the fungus infection of potato started in Belgium and spread from this country to other places in Europe (Bourke, 1964). In Ireland the damage of the potato crop, the main food for the population, was so devastating that thousands of people lost their lives and the others migrated to the U.S.A. (Salaman, 1949). The fact that potato grew in Europe in the absence of *P. infestans* for three centuries before the appearance of the damage of this pathogen indicates that the fungus probably did not travel to Europe with the first introduction of the potato.

The centre of origin of the fungus. *P. infestans* is most probably in Mexico because both mating types (A1 and A2), allowing sexual reproduction, and the greatest genetic variation of resistant *Solanum* species are found in that region (Robertson 1991). As a matter of fact, before 1980, the A1 and A2 mating types were only found in Central Mexico, and in other places of the world, only the A1 mating type appeared to be active. Hohl and Iselin (1984) reported for the first time the presence of the A2 mating type outside the place of origin, in Switzerland in the early 1980s, and later its presence was confirmed in many other countries (Robertson, 1991; and references there in).

The Taxonomy of late blight dates back to 1845. It was first placed in the genus *Botrytis* by Dr. Montagne and later, in the genus *Phytophthora* (De Bary, 1876). Details of the taxonomic history can be found in Waterhouse (1963; 1970).

The hosts range of *P. infestans* is narrow. It can infect potato, tomato and some other *Solanum* species (Turkensteen, 1973). The fungus multiplies in these hosts usually vegetatively, however in the presence of the two mating types, sexual reproduction can occur.

The life cycle of *Phytophthora infestans* starts from the infected tuber or plant tissue where the fungus survives during the winter. The shoots of these tubers contain a living mycelium of *P. infestans* from which sporangiophores emerge bearing sporangia. As a result of rain or sprinkler irrigation the sporangia are washed to the soil to infect other tubers. Leaves touching the infected soil surface or sporangia scattered on the foliage cause more infection of neighbouring plants. Under the condition

of free water from frequent rain or dew and temperatures of 9-25 °C or 0-15 °C direct or indirect germination (releasing zoospores), respectively, of the sporangia occur and infect the living plant tissue. Lesions develop most rapidly at 17-25 °C and take 3-5 days, from where the new sporangia are scattered, for the second cycle of infection (Robertson, 1991). Van der Zaag (1956) showed that a low proportion of infected tubers is capable of carrying the disease from one year to another.

The sexual cycle is based on the presence of both A1 and A2 mating types on the same plant tissue. In that case, it is possible that the hyphae of opposite mating types interact, leading to sexual processes and oospore formation. The oospores are spread to other living tissues and enter the second cycle of infection or to the soil, where they can survive the winter. The oospores can survive in the soil, the environmental stress and can easily start germination to colonise the plant when the conditions are favourable (Drenth, 1995). Sexual reproduction is an alternative quick way of increasing genetic variability in order to develop new races which can both overcome the resistance of the host plant and develop resistance to fungicides.

Early **breeding programmes** were focused on the introduction of resistance from the hexaploid wild species *S. demissum* into the cultivated potato. As a result of the research of Black et al. (1953) and Malcolmson and Black (1966), 11 resistance genes (*R*-genes) were identified and introduced either as a single gene or as multiple genes to *S. tuberosum*.

The resistance was based on single dominant genes and appeared to be race specific. There are strong indications for a gene for gene interaction which was proposed by Flor (1942; 1971). Based on these sources of resistance, 11 *R*-genes to the *P. infestans* isolates were classified. Before 1980, outside the place of origin, the races of *P. infestans* were not complex carrying one or a few virulence factors. But as predicted by Person et al. (1962) the introduction of new *R*-genes caused a selection pressure on the pathogen population resulting in the development of new races capable of breaking the resistance. Because of this phenomenon the breeding with *R*-genes did not provide durable resistance (Turkesteen 1993). Therefore attention was given to use non race-specific resistance in breeding programmes which is expected to be based on polygenes.

Chemical control is another approach to control *P. infestans* by applying sprays either for treatment or for protection based on a forecasting system (Robertson 1991). As a result of this chemical control, *P. infestans* isolates resistant to fungicides appeared (Davidse et al., 1981). Moreover, the application of chemical control is

costly, e.g. in the Netherlands it costs as much as 100 million guilders annually (Davidse et al., 1989). It is also a great source of environmental pollution. After 150 years of intensive research on potato to bring damage caused by *P. infestans* under control, it must be said that it is still threatening world wide, both the agricultural and the economic systems.

Molecular cloning of the earlier mentioned major resistance genes is another approach to understand the mechanism of resistance against this pathogen and may help to reduce the costs of chemical control and environmental pollution.

Isolation of the *R*-genes against *P. infestans* cannot be achieved through m-RNA isolation and cDNA cloning because the gene products are unknown. Therefore, transposon tagging is an alternative method for cloning such genes.

Transposable elements (Transposons) were first described by McClintock (1948; 1951). They are mobile pieces of DNA which move from one place in the genome to another through a process of excision and reinsertion, as a result they can inactivate the expression of a gene by insertion mutation of that particular gene. Transposons occur as families in the form of two types of related elements 1) autonomous, the element is containing a transposase gene so that it can regulate its own excision and reintegration; 2) non-autonomous, the element is not capable for transposition by itself, however, it can be activated by the presence of the autonomous element.

After molecular isolation of transposons it was possible to isolate genes which were mutated by insertion. Through this technique, several genes have been isolated in maize and *Antirrhinum* (Walbot 1992). In maize, the autonomous transposon (*Ac*) was reported to transpose preferentially to closely linked positions (Greenblatt 1984; Dooner and Belachew 1989). This characteristic is advantageous because use of a transposon at a linked position to a particular gene (targeted tagging) increases the chance of mutating that gene by the process of excision and reintegration (Döring 1989). This approach was successfully used in targeted tagging of genes in maize (*R-nj*, Delaporta et al., 1988; *Ts2*, Delong et al., 1993).

Effort has also been made to utilize the well characterized transposons in heterologous plant species like potato, tomato and tobacco. The *Ac* element, as in maize, showed tendency to transpose to a closely linked position in tobacco (Jones et al., 1990; Dooner et al., 1991), Arabidopsis (Keller et al., 1993) and tomato (Osborne et al., 1991) as well as the *Ds* element in tomato (Thomas et al., 1994; Knapp et al., 1994). Also other transposition behaviour was observed in tomato (Healy et al., 1993;

Rommens et al., 1993). Both a random and a targeted tagging approach were successful in heterologous plant species. Through the random tagging approach a male sterility gene in Arabidopsis and a flower colour gene in Petunia were isolated (Aarts et al., 1993; Chuck et al., 1993). On the other hand the targeting tagging approach was used in isolating genes conferring resistance to TMV Virus in tobacco the (*N*-gene), to *Cladosporium fulvum* in tomato (*Cf-9* gene) and to rust in flax (L^6 gene) (Whitham et al., 1994; Jones et al., 1994; Ellis et al., 1995).

Our strategy, to isolate one or more *R*-genes against *P. infestans*, is based on the *Ac-Ds* transposable element system through the targeting tagging approach. The first step is the introduction of the *Ds*-element, through transformation, into a resistant diploid potato clone followed by selection of transformants in which the *Ds*-containing T-DNAs are linked to the resistance gene. This first step can be followed by crossing the selected transgenic plants with *R1r1* or *R1R1* clones and selection for homozygous *R1R1* genotypes still containing the *Ds* element. Introduction of the *Ac* transposase gene to the selected plants activate the *Ds*-element to tag the resistance gene by insertion mutagenesis of *R1* in the third step. The *Ac* transposase will be removed via crossing with the susceptible genotypes (*r1r1*). All the F_1 progeny plants are expected to have the *R1r1* genotype except the tagged mutant which will show susceptibility (*r1::Dsr1*).

Localization of *R*-genes is the first essential step for transposon tagging. For that purpose it is necessary to develop diploid potato populations in which all *R*-genes can be localized. In 1992 the first *R*-gene was mapped (Leonard-Schippers 1992) on chromosome 5. In this study *R1*, *R3*, *R6* and *R7* were localised (Chapter 2 and 3).

High transformation efficiency and fertility are also necessary for the development of hundreds of transposon-containing transformants. Well transforming genotypes that are segregating for *R1* have been developed (chapter 4). Large numbers of transposon-carrying *R1*-resistant transformants have been isolated to optimize the possibility that at least one or a few transformants carrying a *Ds*-containing T-DNA insert near to the position of *R1*-gene can be isolated. Difficulties in isolating the flanking sequences and mapping of the T-DNAs are discussed in chapter (5). Beside these understanding of inheritance and expression of *R*-genes is essential to carry out this type of research. Indications have been found that a second genetic factor might be involved in the expression of *R*-genes. The expression of *R1* appeared to be dependent on the absence of a suppressing factor (chapter 6).

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Chapter 2

Segregation analysis and RFLP mapping of the *R1* and *R3* alleles conferring race-specific resistance to *Phytophthora infestans* in progeny of dihaploid potato parents

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Abstract

Phytophthora infestans (Mont.) de Bary is the most important fungal pathogen of the potato (*Solanum tuberosum*). The introduction of major genes for resistance from the wild species *S. demissum* into potato cultivars is the earliest example of breeding for resistance using wild germplasm in this crop. Eleven resistance alleles (*R* genes) are known, differing in the recognition of corresponding avirulence alleles of the fungus. The number of *R* loci, their positions on the genetic map and the allelic relationships between different *R* variants are not known, except that the *R1* locus has been mapped to potato chromosome 5. The objective of this work was the further genetic analysis of different *R* alleles in potato. Tetraploid potato cultivars carrying *R* alleles were reduced to the diploid level by inducing haploid parthenogenetic development of *2n* female gametes. Of the 157 isolated primary dihaploids, 7 set seeds and carried the resistance alleles *R1, R3* and *R10* either individually or in combinations. Independent segregation of the dominant *R1* and *R3* alleles was demonstrated in two F_1 populations of crosses among a dihaploid clone carrying *R1* plus *R3* and susceptible pollinators. Distorted segregation in favour of susceptibilty was found for the *R3* allele in 15 of 18 F_1 populations analysed, whereas the *R1* allele segregated with a 1:1 ratio as expected in five F_1 populations. The mode of inheritance of the *R10* allele could not be deduced as only very few F_1 hybrids bearing *R10* were obtained. Linkage analysis in two F_1 populations between *R1, R3* and RFLP markers of known position on the potato RFLP maps confirmed the position of the *R1* locus on chromosome 5 and localized the second locus, *R3*, to a distal position on chromosome 11.

Introduction

There are certain features which are common to the genes controlling race-specific resistances to fungal pathogens of crops like maize, barley and flax, that have been carefully characterised. These are: that (1) large numbers of dominant resistance alleles exist that differ in the recognition of the corresponding avirulence alleles of the fungus; and (2) groups of resistance alleles have been mapped to the same region on the genetic map, either as variants at the same or tightly linked loci (Saxena and Hooker 1968; Giese et al., 1981; Jahoor and Fischbeck 1987; Mayo and Sheperd 1980; Islam et al., 1989). For the host-pathogen interaction between the potato and *Phytophthora infestans* (Mont.) de Bary, 11 resistance or *R* genes have been identified so far, originating from the hexaploid wild species *S. demissum* (Black et al., 1953; Malcolmson and Black 1966; Ross 1986; Wastie 1991). The phenotype of the resistant alleles is a hypersensitive resistance reaction that takes place after infection by specific races of the fungus. When analysed, the *R* alleles have shown Mendelian inheritance as single dominant factors (Mastenbroek 1953; Malcolmson and Black 1966) and their relationship with the pathogen conforms to the gene-for-gene hypothesis of Flor (1942; 1971). Allelism among *R* alleles, linkage relationships, and map positions of the genetic loci involved are not known, with the exception of the *R1* locus which has been located on chromosome 5 of the potato RFLP map (Leonards-Schippers et al., 1992). It is, therefore, of interest to determine the genomic positions of other *R* loci relative to *R1* and to test whether or not *R* loci of potato are structured similarly to multiallelic resistance loci of other plant species.

One or more *R* alleles are present in many tetraploid potato varieties. They provided the first resistance phenotype against *P. infestans*, which causes the late blight of potato, one of the most destructive fungal diseases of this crop. They are the earliest examples of breeding for resistance in potato based on the introduction of germplasm from wild *Solanum* species, in the case of the late blight of potato, from *S.demissum* (Ross 1986; Wastie 1991). Tetraploid potato varieties (2n=4x=48) known to carry *R* alleles were reduced, therefore, to the diploid level (Hermsen and Verdenius 1973) in order to obtain dihaploid parents (2n=2x=24) harbouring heterozygous *R* alleles which could be then used in crosses for the production of segregating F_1 populations. We have analysed such progeny segregating for specific *R* alleles and RFLP markers having known positions on the RFLP map of potato (Gebhardt et al., 1991; 1993). The analysis verified the map position of the *R1* locus

(Leonards-Schippers et al., 1992) and revealed the existence and chromosomal position of a second locus, *R3*, in the potato genome.

Materials and methods

Origin of plant material

Ten cultivars reported to have one or more *R* alleles were selected from the Dutch variety list (Anonymous 1989). Dihaploids (2n=2x=24) were induced through prickle pollination using the diploid species, *S.phureja*, clones IVP 35 and 48 (Hermsen and Verdenius 1973) and IVP 101 (Hutten et al., 1990). The dihaploids obtained were tested with *P. infestans* race 0. The resistant dihaploids were grafted onto tomato root stock (cv. Virosa) for flower induction and pollination. The diploid clones 87-1017-5, 87-1024-2, 87-1029-31 and 87-1031-29 (Jacobsen et al., 1989) and J89-5002-6, J89-5002-18, J89-5040-1 and J89-5040-2 were used as pollinators. They were fertile and susceptible to *P. infestans (rr)*.

Determination of fertility

Female fertility of the primary dihaploids and selected F_1 hybrids was determined after pollination with fertile diploids by berry set and seed set per berry. Male fertility was estimated after staining pollen with lactophenol acid fuchsin.

Inoculum

The *P. infestans* races 0, 1, 3.7, 4.10, 1.4.10, 1.3.4.7, 1.3.4.7.10, 1.3.4.10.11 and 3.4.7.8.10.11 were kindly supplied by L.J. Turkensteen (Research Institute for Plant Protection, IPO-DLO, Wageningen) and F. Govers (Department of Phytopathology, Wageningen Agricultural University). The isolates were maintained on tuber slices of cv. Bintje to ensure sufficient pathogenicity on foliage. The tuber slices were kept in moist trays in a dark climate chamber at 17° C. For preparation of the inoculum, fresh tuber slices were infected with a spore suspension. After 6 days, spores were collected by washing the slices with demineralised water. The concentration of the resulting spore suspension was adjusted to 10^4 conidia/ml.

Inoculation procedure

Plants (8- to 10-weeks-old) were tested using the detached leaflet technique (Toxopeus 1954; Mooi 1965; Umaerus 1969). For inoculation, fully expanded leaves were

collected and fixed upright by their petioles in floral foam (Smithers-Oasis) saturated with water in a container with a transparent cover. A thin film of inoculum was sprayed on the lower surface of the leaves using a hand sprayer fitted with a fine nozzle. The containers were kept in a climate chamber at 17° C in 16 h light/8 h dark. The leaves were kept in ca. 100% relative humidity for 24 h, then ventilated to allow the leaves to dry. The disease assessment was carried out 5 days after inoculation. Plants were scored as: (1) resistant (R) when symptoms were absent or a hypersensitive reaction was visible; and (2) susceptible (S) when sporulating lesions developed. The resistance test was repeated twice. Parents, as well as defined genetic material resistant to specific races of *P. infestans* (C. Mastenbroek unpublished: tetraploid tester clones containing *R0; R1, R2, R3* and *R10*, and *R1 R3* loci), were used as controls in both tests.

RFLP analysis

Total genomic DNA was extracted from 0.3-0.4g freeze-dried leaves and shoots of parental lines and F_1 hybrids as described previously (Gebhardt et al., 1989). DNA was purified by CsCl gradient centrifugation in all cases. Restriction digests, electrophoresis, electrotransfer to Nylon membranes (Amersham, Hybond N) and hybridization to RFLP marker probes were carried out according to Gebhardt et al. (1989). Linkage analysis among *R1, R3* and RFLP alleles was performed using computer programs based on the models and algorithms given by Ritter et al. (1990). Marker probes were selected according to their map position on an updated version of the potato RFLP map (Gebhardt et al., 1991; 1993). GP markers originated from genomic DNA of potato, and CP markers were derived from potato cDNA clones. The marker probe TG105 from tomato was provided by S.D. Tanksley (Cornell University, Ithaca, NY, USA). Small letters in parenthesis indicate that more than one RFLP locus is detected with the same probe.

Results

Isolation and characterisation of dihaploids

From ten cultivars bearing one or more *R* alleles, 157 parthenogenetic dihaploids were obtained. Some of them were malformed and of low vigour. All dihaploids were tested for resistance to *P. infestans* race 0, and 60 were resistant (Table 1). Assuming unbiased segregation of *R* alleles in dihaploids derived from tetraploid varieties, a

Table 1 Number of dihaploids isolated from ten cultivars and their reaction to *Phytophthora infestans* race 0

Cultivar	Number of dihaploids			
	Total	Test with race 0		X^2 1:1
		Resistant	Susceptible	
R1				
Maritta	2	0	2	nt
Morene	39	2	37	31.40**
Saturna	6	4	2	nt
Total	47	6	41	26.06**
R3				
Cardinal	6	2	4	nt
Eba	30	8	22	6.53*
Radosa	28	6	22	9.14**
Total	64	16	48	16.00**
R1R3				
Astarte	3	0	3	nt
Atrela	2	0	2	nt
Total	5	0	5	nt
R1R3R10				
Hertha	22	20	2	nt
R1R2R3R10				
Escort	19	18	1	nt
Total	157	60	97	

nt, not tested
*, **, significant at $P = 0.05$ and 0.01, respectively

segregation ratio 1:1 (resistant versus susceptible) is expected for one *R* allele in the simplex constitution and of 5:1 in the duplex constitution. In varieties with two unlinked *R* genes both with one dominant allele, a 3:1 (both simplex) or an 11:1 (one simplex, one duplex) segregation ratio of resistant versus susceptible dihaploids is expected. Even higher frequencies of resistant dihaploids should be observed when more than two genes, each with one *R* allele, are present in the tetraploid variety.

The frequency of resistant dihaploids was much lower than expected in the dihaploid progeny of cvs. Morene, Eba and Radosa (Table 1). Also the five dihaploids extracted from cvs. Astarte and Atrela bearing two *R* alleles were, contrary to expectation, susceptible. On the other hand, most of the dihaploids derived from cvs. Escort and Hertha carrying four and three *R* alleles, respectively, were resistant. Due to the low numbers of dihaploids obtained, significant segregation ratios could not be determined for most of the sets of primary dihaploid progeny. Pooling the numbers of dihaploids of cultivars with only one *R* allele shows, nevertheless, a highly significant bias towards susceptibility (Table 1).

After grafting onto tomato rootstocks, 24 of the 60 resistant dihaploids flowered normally. More than 100 berries were set after pollination of about 400 flowers using different susceptible diploid pollinators. Berries as well as seeds were obtained from seven well-flowering dihaploids. They originated from cvs. Cardinal (CarEP.1; CarEP.8), Escort (Esc.42), Hertha (Her.44; Her.64), Radosa (RadEP.90) and Saturna (J90-6026-4). Seeds per berry varied among these dihaploid genotypes from 1.3 to 12.7. This indicates that 7 out of 157 primary dihaploids showed potential value for developing populations segregating for one or more *R* alleles. These seven dihaploids were tested with additional races of *P. infestans* to determine which of the *R* alleles were present. J90-6026-4 and Her.44 contained *R1*, CarEP.1, CarEP.8 and RadEP.90 contained *R3* and the dihaploids Esc.42 and Her.64 contained *R1R3* and *R1R3R10R?*, respectively (Table 2). The *R2* and *R10* alleles present in the cv. Escort were not transferred to Esc.42. A remarkable observation was that cv. Hertha was susceptible to race 1.3.4.7.10, whereas the dihaploid Her.64 was resistant, indicating the expression of a new *R* specificity in Her.64.

Seed set and segregation of F_1 plants

Both male and female fertility of resistant primary dihaploids was low. Observations on F_1 hybrids in crosses among the dihaploids and susceptible pollinators indicated that flowering capacity and fertility increased. This was evident from the number of seeds

Table 2 Segregation in the F_1 hybrids of resistant dihaploids crossed with susceptible diploids, after inoculation with *P. infestans* race 0.

Dihaploid resistant Parent	F_1 population (Code)	Test with race 0 Number of plants			
		R	S	X^2	
				1:1	3:1
R1					
J90-6026-4	J91-6146	10	15	1.00	
Her.44	J91-6166	1	3	nt	
R3					
CarEP.1	J91-6152...				
	J91-6155	2	34	28.44**	
CarEP.8	J91-6156...				
	J91-6162	12	128	96.11**	
RadEP.90	J91-6170	7	12	1.32	
J91-6162-27	J92-6454	20	26	0.78	
R1R3					
Esc.42	J91-6164	58	23	15.12**	0.50
	J91-6165	14	15	0.03	11.05**
R1R3R10R?					
Her.64	J91-6167...				
	J91-6169	39	52	nt	

nt, not tested
**, significant at $P = 0.01$

per berry that increased from 1.3-12.7 in parental dihaploids to 28.4-77.3 in the F_1 hybrids.

Twenty sets of progeny were classified into susceptible (S) and resistant (R) plants using *P. infestans* race 0 as inoculum (Table 2). Nineteen sets resulted from crosses of the seven primary dihaploids with susceptible clones as pollinators. One set of progeny (J92-6454) originated from a cross between the F_1 hybrid J91-6162-27(*R3r*) of progeny J91-6162 and the susceptible clone 87-1024-2 (*rr*).

The 1:1 ratio expected for the segregation of the *R1* and *R3* alleles present in

the heterozygous state was found in populations J91-6146 (*R1*), J91-6170 and J92-6454 (*R3*). Population J91-6166 was too small to assess the segregation ratio. The sets of progeny resulting from crosses with dihaploids CarEP.1 and CarEP.8 of cv. Cardinal showed a highly distorted ratio for *R3* in favour of susceptibility (Table 2). The two progeny sets J91-6164 and J91-6165 had the female parent Esc.42 in common, which carries *R1* plus *R3* and they differed only for the susceptible male parent. Assuming no linkage between *R1* and *R3*, a 3:1 segregation ratio (resistant versus susceptible) was expected and, indeed, found for progeny J91-6164. Progeny J91-6165 segregated, however, with a 1:1 ratio, compatible with *R1* and *R3* being closely linked in coupling phase (Table 2). Allelism between *R1* and *R3* was excluded because susceptible F_1 plants were found. The segregations observed in the progeny of Her.64 which, based on inoculation data, should have carried at least *R1*, *R3* and *R10*, did not fit the expectation of at least two unlinked *R* loci, as too many susceptibles were found.

The populations J91-6164, J91-6165, J91-6167, J91-6168 and J91-6169 were further tested with a number of specific races (0, 1, 3.7, 4.10, 1.4.10, 1.3.4.7, 1.3.4.10.11, 1.3.4.7.10, and 3.4.7.8.10.11) in order to determine the presence of *R1*, *R3*, *R10* and combinations thereof in resistant F_1 individuals (Table 3). This analysis demonstrated again that *R1* and *R3* were unlinked in the populations J91-6164 and J91-6165. A regular segregation pattern of 1:1:1:1 for the phenotypes R1,R3,R1R3 and susceptibles (S) was found in the progeny J91-6164 as expected for the segregation of two unlinked *R* loci, but not in progeny J91-6165. In this latter progeny, the segregation was distorted for *R3*, with an excess of susceptible plants (6 R3 versus 22 R1 or S, $X^2_{1:1}=9.14$, $P<0.01$)whereas the *R1* allele segregated as expected (10 R1 versus 18 R3 or S, $X^2_{1:1}=2.28$, $P>0.05$). The mode of inheritance of *R10* could not be deduced from populations J91-6167, J91-6168 and J91-6169, as only 2 out of 38 resistant plants had the R10 phenotype. The same was true for *R3* in these three crosses: only five plants expressed the *R3* allele (Table 3). The *R1* allele segregated, however, as expected in progeny J91-6167 (22 R1 versus 31 S, $X^2_{1:1}=1.18$, $P>0.2$) and J91-6168 (15 R1 versus 15 S, $X^2_{1:1}=0.0$, $P>0.8$). One plant of progeny J91-6167, J91-6167-35, showed the same resistance pattern as the mother plant Her.64 (Table 3).

RFLP mapping of R1 and R3

The progeny J91-6164 (Table 3), originating from the cross Esc.42 (*R1r/R3r*)x87-1031-29 (*rr/rr*), was chosen in order to identify the positions of *R1* and *R3* on the

Table 3 Segregation of *R* alleles in the F_1 hybrids of ESc.42 (*R1R3*) and Her.64 (*R1R3R10R?*) crossed with susceptible diploids.

Populations	Number of plants with phenotype[a]						
	R1	R3	R1R3	R1R3R10	S	Not tested	X^2 1:1:1:1
R1R3							
J91-6164	14	18	20	--	23	1	2.38
J91-6165	7	3	3	--	15	1	13.71**
R1R3R10R?							
J91-6167	19	--	2	1[b]	31	--	nt
J91-6168	13	--	1	1	15	--	nt
J91-6169	1	--	--	--	6	--	nt

nt, not tested

**, significant at $P=0.01$

[a] Other possible combinations between *R* alleles were not found

[b] This plant shows a resistance behaviour similar to that of the mother plant Her.64

RFLP linkage map of potato. Fifty-four plants (10 R1, 14 R3, 12 R1R3, 18 S) and the parents were analysed with 83 marker probes, chosen based on their positions on the 12 potato linkage groups, for cosegregation of RFLP alleles with the *R1* and the *R3* resistance alleles, respectively. Forty percent of all the markers tested were not informative, being either homozygous in the resistant parent Esc.42 or not polymorphic between both parents.

The map position of the *R1* locus on chromosome 5, which was determined previously (Leonards-Schippers et al., 1992), was confirmed by testing the marker *GP21*, known to be closely linked to *R1* (2.5cM). In progeny J91-6164, *R1* was linked (4.5cM away) to the same marker locus (data not shown). The *R3* locus was identified at a distal position on chromosome 11 (Fig. 1B). For comparison, an updated version of chromosome 11, as deduced from segregation data for 12 RFLP marker loci informative in the cross used for constructing our first RFLP map of potato (Gebhardt et al., 1991; 1993), is shown in Fig. 1A. The genetic distance between *R3* and the proximal group of three closely linked RFLP loci *GP250(a)*, *GP185* and *TG105(a)* was 7cM. The most closely linked RFLP locus, *GP35(k)*, was not useful for the identification of the map position of *R3*, as the position of *GP35(k)* itself was unknown. The marker probe GP35 detects multiple loci in the potato genome (Gebhardt et al., 1989) only some of which show segregating RFLP alleles in any specific cross. *GP35(k)* was not detected in the cross used for constructing the chromosome 11 map shown in Fig. 1A.

The position and the order of *R3* and of the RFLP loci linked to it were confirmed by segregation analysis using 41 plants and the parents of progeny J92-6454 (Table 2). The locus *R3* was found to be 2.4cM (one recombinant) distal to *GP250(a)* and *TG105(a)* and only 5cM distal to *GP38* (Fig. 1C), whereas in progeny J91-6164 the distance between *R3* and *GP38* was 29 cM, nearly 6 times larger. The recombination behaviour was, therefore, very different in populations J91-6164 and J92-6454. When compared with the recombination among the same marker loci in the RFLP mapping population shown in Fig. 1A, the cross J91-6164 showed increased, and the cross J92-6454 reduced recombination in the resistant parent.

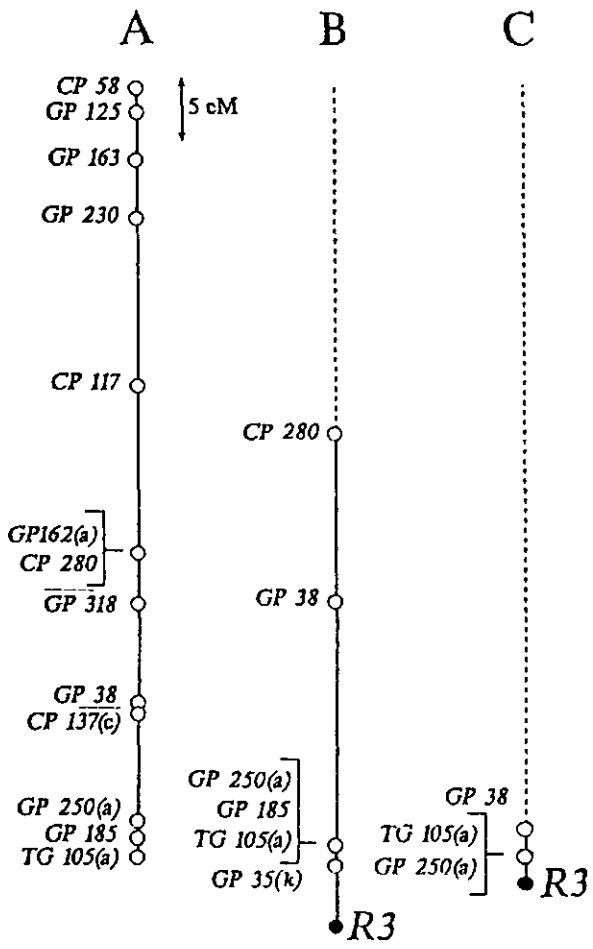

Fig 1A--C. Map position of the *R3* locus on chromosome 11 of potato. **A** Chromosome 11 linkage map deduced from RFLPs segregating in the mapping population of Gebhardt et al. (1989; 1991). **B** Part of chromosome 11 with the *R3* locus as derived from RFLP analysis in progeny J91-6164. **C** Part of chromosome 11 with the *R3* locus as derived from RFLP analysis in progeny J91-6454.

Discussion

Only 7 out of 157 dihaploids extracted from tetraploid varieties expressing *R* alleles proved to be both fertile and resistant to specific races of *P. infestans*. The low efficiency was due to the low fertility and the preferential recovery of susceptible dihaploid plants. Low vigour and low fertility is an observation that holds for a large proportion of primary dihaploids of potato (Carroll and Low 1975; 1976; Ross 1986). It is explained as the phenotypic manifestation in the dihaploids of unfavourable recessive alleles present in the tetraploid. Such unfavourable alleles might also be particularly linked to the *R* alleles present in cultivated varieties which have been introduced from the wild species *S.demissum*. Although diluted by backcross breeding, considerable amounts of wild germplasm should still be present around the *R* loci (Stam and Zeven 1981). Linkage drag may be, therefore, the reason for the low frequency of resistant and fertile dihaploids recovered. Fertility improved in F_1 hybrids of the primary dihaploids crossed with fertile plants. This is in accordance with previous observations (Ross 1986 and references therein).

Segregation analysis of the alleles *R1* and *R3* in a series of F_1 populations originating from crosses of resistant with susceptible fertile diploids revealed that, in the majority of cases, the 1:1 ratio expected for the segregation of a single, dominant allele in heterozygous condition was highly distorted towards susceptibility. The *R3* allele was more affected than the *R1* allele: of the 18 populations segregating for *R3*, all but 3 (J91-6170, J91-6164, J92-6454; Table 2) exhibited the distortion, whereas in all 5 progeny segregating for *R1* (J91-6146, J91-6164, J91-6165, J91-6167, J91-6168) a normal 1:1 segregation ratio was found. Normal segregation of *R3* depended on the pollinator used, as becomes evident on comparing populations J91-6164 and J91-6165, which shared the female parent. Distorted segregation ratios are frequently observed in crosses of dihaploid potato lines (Gebhardt et al., 1991) and can be explained by postzygotic selection against unfavourable allelic combinations. In the crosses analysed, such unfavourable allelic combinations might have been present at the *R3* locus itself or at loci linked to *R3*.

RFLP analysis of the F_1 populations J91-6164 and J92-6454 confirmed the map position of *R1* and revealed that of the *R3* locus on the genetic map of potato. The location of *R1* on chromosome 5 (Leonards-Schippers et al., 1992) is supported by the close linkage of the *R1* allele segregating in progeny J91-6164 to the marker locus *GP21* on chromosome 5. The *R3* locus was found at a distal position on chromosome

11 linked to a proximal group of three marker loci *TG105(a)*, *GP185*, and *GP250(a)*. The tomato marker probe TG105 detects homoeologous RFLP loci on chromosome 11 of *Lycopersicon* and *Solanum*, whose genomes are homoeologous (Tanksley et al., 1992).

In tomato, the marker locus *TG105* is closely linked (0.4cM) to the *Fusarium oxysporum* resistance locus *I2* (Segal et al., 1992). The genetic distance of 0.4cM was found to be equivalent to a physical distance of approximately 17 kb only (Segal et al., 1992). The *P. infestans* resistance locus *R3* in potato is linked to the same marker locus, however, depending on the progeny used for mapping, at genetic distances of 7 cM and 2.4 cM, respectively. The physical distance corresponding to this genetic distance is not known at present in the potato, which has about the same genome size as tomato (determined by flow cytometry, K.M. Blok, personal communication). It seems likely, however, that *R3* is also physically more distant from *TG105* in potato than *I2* in tomato. The large difference between recombination frequencies separating the *R3* and *TG105* loci in two different crosses points to the fact that the correlation between genetic and physical distance is already highly variable within a single species. It depends not only on the position in the genome of the loci considered (Segal et al., 1992; Ganal et al., 1989) but also on the specific cross used in mapping experiments. Similar observations have been made in other plant species, for example in barley (Görg et al., 1993).

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Chapter 3

Genetic analysis and RFLP mapping of *R6* and *R7* loci of potato conferring race-specific resistance to *Phytophthora infestans* (Mont.) de Bary.

Abstract

Resistant differential strains of the tetraploid *Solanum tuberosum*, clones MaR6 and MaR7, were used as parental plants for the parthenogenetic induction and selection of diploid genotypes containing the *R6* and *R7* resistance loci to *Phytophthora infestans*. A resistant dihaploid from MaR7 could directly be used as crossing parent to produce a diploid F_1 progeny suitable for genetic and RFLP analysis. MaR6 did not produce directly useful dihaploids. After crossing of MaR6 with a tetraploid susceptible genotype, sufficient resistant F_1 clones were selected. In this F_1 population a 5:1 segregation was found indicating a duplex genotype for *R6* in MaR6. The resistant genotypes were then used as parents for the induction of suitable dihaploids. Several resistant dihaploids bearing *R6* could be crossed with a diploid susceptible genotype. The diploid populations, segregating for *R6* or *R7*, generally showed an excess of susceptible plants. RFLP analysis using probes linked to either *R1* or *R3* showed clear linkage of both *R6* and *R7* to the same probes that were linked to *R3* on chromosome 11.

Introduction

In potato, the resistance to *Phytophthora infestans* is either horizontal or vertical. The horizontal form seems to be polygenic (Umaerus et al., 1983; Wastie 1991). The vertical resistance, however, is controlled by major dominant factors, called *R*-genes (Mastenbroek 1953; Malcomson and Black 1966). *R*-genes were introduced from different accessions of the hexaploid wild species *Solanum demissum* into the cultivated tetraploid potato by repeated back crossing. They confer race-specific resistance to *P. infestans*. Eleven *R*-genes are known and used in breeding so far (Malcomson and Black 1966; Shaw 1991). Therefore, eleven tetraploid resistant potato genotypes, obtained by backcrossing of potato-*S. demissum* hybrids with potato, and expressing *R*-genes from different accessions, are used as differentials for the characterization of isolated unidentified races of *P. infestans* (Turkensteen pers. comm.).

Molecular isolation of these *R*-genes would facilitate understanding of the resistance mechanism to the fungus. Here we have used the differentials as a source

for localization of *R*-genes on the map of potato. Mapping of *R*-genes, expressed in the differentials, is the first step for either transposon tagging or map based cloning. The resistance factors *R1* and *R3* were recently mapped (Leonards-Schippers et al., 1992; El-Kharbotly et al., 1994) on chromosome 5 and 11 respectively using RFLP markers with known positions on the RFLP map of potato (Gebhardt et al., 1991; 1994). Here, we have determined the position of both factors *R6* and *R7* in the potato genome by analyzing segregating populations at the diploid level. For this purpose, dihaploids were isolated by haploid parthenogenesis (Hermsen and Verdenius, 1973) from differential tetraploid genotypes containing *R6* an *R7*. This approach was followed by crossing of dihaploids with susceptible diploids for segregation studies of these *R*-genes and application of molecular probes of the earlier described potato map (Gebhardt et al., 1991).

Materials and methods

Plant material

The tetraploid potato clones Ma*R6* and Ma*R7*, resistant to specific races of *P. infestans* (C. Mastenbroek unpublished: tetraploid differential tester clones containing *R6* and *R7* factors, respectively), were used to develop diploid populations suitable for RFLP mapping. They were kindly supplied by Dr. L.J. Turkensteen (Research Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands). From these clones dihaploids (2n=2x=24) were induced through prickle pollination using the diploid species, *Solanum phureja*, clones IVP 35 and 48 (Hermsen and Verdenius 1973) and IVP 101 (Hutten et al., 1994). The dihaploids obtained were tested with *Phytophthora infestans* race 0. The resistant dihaploids were grafted onto tomato root stocks of *cv.* Virosa for flower induction and pollination. The fertile diploid clones 87-1024-2 (Jacobsen et al., 1989) and the amylose-free tetraploid clone J90-6001-3 (Flipse et al., 1995), both were susceptible to *P. infestans* (*rrrr*), were used as pollinators.

Screening for resistance

The genotype of Ma*R6* and Ma*R7* was confirmed using different races of *P. infestans*. The same test was carried out for all susceptible pollinators and F1 progenies. The races of *P. infestans* were kindly supplied by Dr. L.J. Turkensteen (Research Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands) and Dr. F. Govers

(Department of Phytopathology, Wageningen Agricultural University, The Netherlands).

Preparation of *P. infestans* inoculum (race 0, 7, 2.7, 1.3.4.7 and 1.2.3.6.7) and the inoculation procedure were carried out as previously described (El-Kharbotly et al., 1994).

Ploidy level determination

Ploidy level of the potential dihaploid seedlings was determined by counting the number of chloroplasts in the stomatal guard cells (Frandsen 1967)

RFLP analysis

Total genomic DNA was extracted from 0.3-0.4 g freeze dried leaves and shoots of parental lines and F_1 hybrids as described previously (Gebhardt et al., 1989). DNA was purified by CsCl gradient centrifugation in all cases. Restriction digests, electrophoresis, electrotransfer to Nylon membranes (Amersham, Hybond N) and hybridization to RFLP marker probes were carried out according to Gebhardt et al., (1989). Linkage analysis among *R6, R7* and RFLP alleles was performed using computer programs based on models and algorithms given by Ritter et al., (1990). Marker probes were selected according to their map position on an updated version of the potato RFLP map (Gebhardt et al., 1991; 1994). GP markers originated from genomic DNA of potato, and CP markers were derived from potato cDNA clones. Small letters in parenthesis indicate that more than one RFLP locus is detected with the same probe.

Results

Development of populations for mapping of R-genes

The resistant genotypes, containing *R*-gene(s), and the susceptible ones are expected to show an incompatible (hypersensitive) and compatible reaction (sporulating lesions) respectively, after inoculation with *P. infestans* race 0. On the other hand, the resistant clones are expected to show a compatible reaction (susceptibility) after inoculation with races containing the virulence factor corresponding with the resistance gene. The reaction pattern of Ma*R6* and Ma*R7* and of pollinators, with different races of *P. infestans*, was as expected (Table 1, A).

Table 1 Reaction of susceptible, *R6* and *R7* diploid potato clones on different races of *Phytophthora infestans*. These clones were used in different crosses.

Potato clones	Genotypes	Races of *Phytophthora infestans*				
		0	7	2.7	1.3.4.7	1.2.3.6.7
A:						
Expected reaction of:	*R6*---	R	R	R	R	S
	R7---	R	S	S	S	S
Observed reaction of:						
Ma*R6*	*R6R6r6r6*	R	R	R	R	S
Ma*R7*	*R7r7r7r7*	R	S	S	S	S
Male Parents:						
S.phureja	*r r*	S	S	S	S	S
J90-6001-3	*r r r r*	S	S	S	S	S
87-1024-2	*r r*	S	S	S	S	S
B:						
Female parent J92-6442-8	*R6r6*	R	R	R	R	S
Population* J92-6601	*R6r6*	R	R	R	R	S
	r6r6	S	S	S	S	S
Female parent BE93-4053-6	*R7r7*	R	S	S	S	S
Population* BE94-4101	*R7r7*	R	S	S	S	S
	r7r7	S	S	S	S	S

R= resistant; S= susceptible.
*, Population originating from either the female parent J92-6442-8 or BE93-4053-6 after crossing with the susceptible male parent 87-1024-2.

The production of dihaploids from clone Ma*R6* was low because of its short flowering period. From the interspecific cross using *S. phureja*, three dihaploids were isolated. All appeared to be resistant to *P. infestans* (Table 2), but they either did not flower or produced flower buds aborting before or after pollination. To solve the problem of sufficient dihaploid induction, the tetraploid Ma*R6* clone was crossed with the tetraploid susceptible clone J90-6001-3. The F_1 progeny (J91-6145) was analyzed for segregation of the *R6* factor. Forty five F_1 genotypes were tested with race 0. A segregation ratio (resistance versus susceptible) of 1:1 or 5:1 was expected in case that *R6* is present in simplex (*R6r6r6r6*) or duplex (*R6R6r6r6*) form respectively. A segregation of 39 resistant to 6 susceptible genotypes was found which fit with 5:1 ratio ($X^2=0.36$, $P>0.5$) indicating that *R6* in Ma*R6* is in duplex form. Induction of dihaploids from the J91-6145 population was easier than from MaR6 because of a

Table 2 Number of dihaploids isolated from tetraploid genotypes with defined *R*-genes and their reaction to *Phytophthora infestans* race 0.

Genotype	Number of dihaploids			Crossable resistant dihaploids (Code)
	Total	Test with race 0		
		R	S	
R6				
Ma*R6*	3	3	0	
J91-6145-2*	6	4	2	J92-6435-1
J91-6145-4	6	5	1	
J91-6145-6	5	2	3	
J91-6145-7	17	12	5	J92-6441-1, 2 J92-6442-1, 8, 10
J91-6145-8	8	6	2	
Total	42	29	13	
R7				
Ma*R7*	6	2	4	BE93-4053-6

*, F_1 progeny plants containing *R6* from the crossing Ma*R6* X J90-6001-3

longer flowering period and high berry set. Forty two dihaploids were isolated from crosses of five selected tetraploid genotypes which were resistant (Table 2). Twenty nine of them appeared to be resistant. They were grown to flowering stage and tested for seed set after pollinating with the susceptible clone 87-1024-2 (Table 2).
MaR7 did not need an additional crossing cycle at the tetraploid level because the isolated resistant dihaploids did flower and produce sufficient F_1 seed in 2x X 2x crosses. Six dihaploids were isolated from which 2 of them showed resistance to race 0 (Table 2).

Segregation of F_1 progeny

Despite of the low male and female fertility of the dihaploids, a few resistant dihaploids set seed. Six and one diploid populations, segregating for either *R6* or *R7* respectively, were obtained. They were screened for resistance to *P. infestans* using inoculum of race 0. Four F_1 progenies J92-6589, J92-6595, J92-6601 and BE93-4035 segregating for *R6* showed a 1:1 ratio at P=0.05 but J92-6590 and the combined results in the homogeneity test showed excess of susceptible genotypes (Table 3).
A 1:1 segregation ratio was expected for the F_1 population containing the *R7* locus. However a high frequency of susceptible genotypes was also observed in this population (Table 3).

The populations J92-6601 and BE94-4101 were selected for RFLP analysis. The parental clones and the F_1 populations were tested with specific races of *P. infestans*. These tests confirmed the presence of either *R6* or *R7* in J92-6601 and BE94-4101 populations respectively (Table 1-B).

RFLP mapping of R6 and R7

The progenies J92-6601, BE94-4101 and their parents (Table 3) were analyzed for cosegregation of RFLP alleles with the *R6* and the *R7* resistance loci respectively. In the first pilot experiments the RFLP alleles which are linked to either *R1* or *R3* (Leonards-Schippers et al., 1992; El-Kharbotly et al., 1994) were tested.

Cosegregation was not detected when the marker *GP21*, which is linked to *R1* was used whereas the two RFLP loci *GP250(a)* and *GP185(a)* closely linked to *R3* did cosgregate with both *R6* and *R7*. In the progeny BE94-4101, segregating for *R7*, only one recombinant was found in the class of susceptible phenotypes. Surprisingly, 12 recombinants were found, in progeny the J92-6601, all in the class of resistant phenotypes. Further analysis using different RFLP alleles confirmed the position and

Table 3 Segregation in the F_1 populations of resistant dihaploids crossed with the susceptible clone 87-1024-2, after inoculation with *P infestans* race 0.

Dihaploid resistant parent	F_1 population (Code)	Test with race 0 Number of plants		
		Resistant	Susceptible	$X^2$1:1
R6				
J92-6435-1	J92-6589	19	24	0.58
J92-6441-1	J92-6590	5	14	4.26*
J92-6442-1	J92-6595	3	9	3.00
J92-6442-8	J92-6601	35	53	3.68
J92-6442-10	BE93-4035	4	7	0.82
Total		66	108	
Homogeneity				10.14^*_{4df}
R7				
BE93-4053-6	BE94-4101	39	64	6.07*

*, significant P=0.05

the order of *R6* or *R7* with molecular markers at the distal part of chromosome 11 (Fig 1 A; B).

Discussion

In potato, efficient localisation of resistance genes to *P. infestans* is highly dependent on the availability of appropriate segregating populations at the diploid level. Earlier research on *R1* and *R3* (El-Kharbotly et al., 1994) clearly showed the phenomenon of distorted segregation which was also found in this study. This seems to be connected with *S. demissum* which is the source of this type of resistance. Distorted segregation was also described for *Rx2* locus conferring resistance to potato virus X (Ritter et al., 1991), *Gpa* locus for nematode resistance (Kreike et al., 1994) and the *P* locus, involved in the production of blue anthocyanins (Van Eck et al., 1993). However, in other studies on introduced nematode resistance (Hutten et al., 1995) and on amylose-

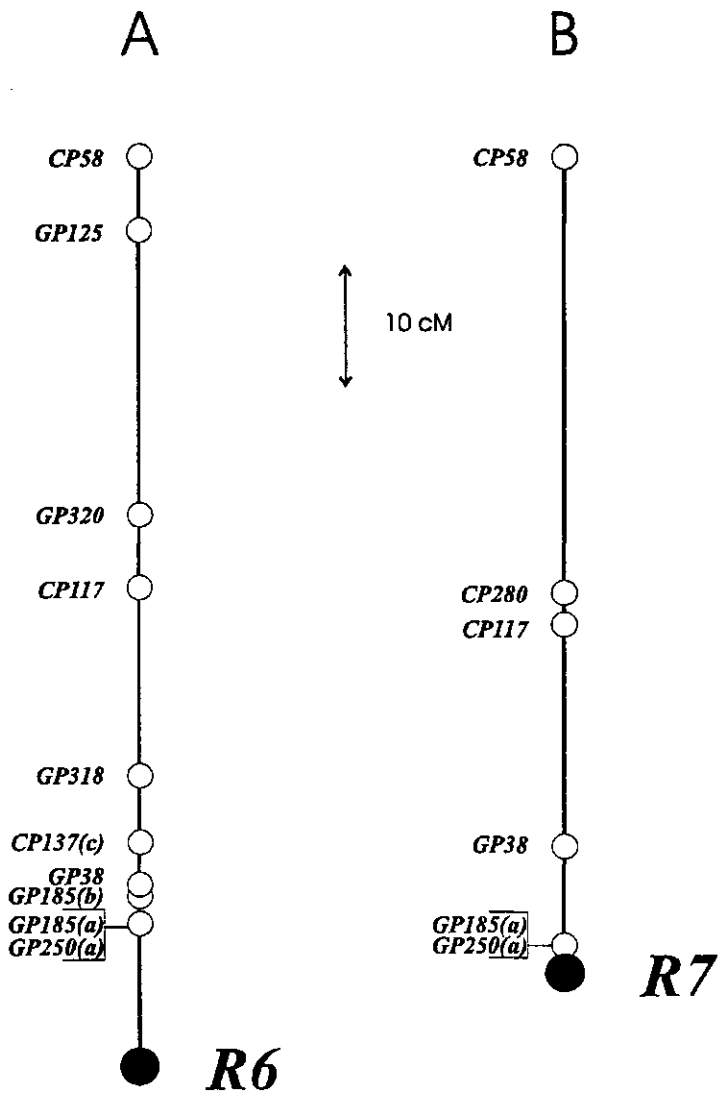

Fig 1. Map positions of A) *R6* and B) *R7* on chromosome 11. Chromosome 11 linkage map deduced from RFLPs segregating in the mapping population of Gebhardt et al. (1989; 1991).

free (*amf*) potato starch (Jacobsen et al., 1989) segregation patterns appeared to be normal. A new complication in the generation of *R6* segregating material was the lack of sufficient flowering of the original tetraploid clone MaR6. Therefore, parthenogenetic progeny consisted of only a few poorly flowering resistant dihaploids which imposed a limitation to obtain a segregating diploid population for molecular mapping. The development of well flowering *R6* resistant tetraploids after crossing with a highly fertile susceptible tetraploid clone as new basic materials for dihaploid induction appeared to be the key factor in order to solve this problem. Most of the (large) diploid populations, segregating for *R6*, showed a 1:1 segregation ratio. However, the pooled data in the homogeneity test indicated a structural excess of susceptible genotypes.

The factors *R6* and *R7*, like *R3* in earlier studies (El-Kharbotly et al., 1994), appeared to be present on the distal position of chromosome 11 with close linkage to the probes GP250 and GP185. A drawback is that all three resistance factors were localised in different populations which made it difficult to determine their relative positions with respect to each other. These resistance factors originated from three parents with different breeding histories. An important observation in this study was the clear difference in recombination frequency between the resistance factors *R6* or *R7* and the closely linked molecular probes. In the population segregating for *R7* a restricted degree of recombination was found, whereas in that for R6 recombination occurred more regularly within the class of resistant phenotypes. However, in the populations segregating for *R3* all recombinations were found within the class of susceptible phenotypes (El-Kharbotly et al., 1994). These differences in recombination behaviour and frequency can reflect the differences in physical distance between these factors or might be the result of the introgression of different sizes of chromosome inserts of *S. demissum* in the potato genome (Stam and Zeven 1981). This is expected to be visualized by chromosome painting based on genomic DNA *in situ* hybridization (GISH) (Jacobsen et al., 1995). In our laboratory attempts are being made to develop a chromosome painting method for the detection of alien introgressions between *S. tuberosum* and *S. demissum* using species specific repetitive DNA probes. In case of existence of different physical distances between the *R*-loci and the RFLP alleles it must be possible to combine all three factors on one and the same chromosome. On the other hand the expectation that different pieces of the *S. demissum* chromosome containing *R3*, *R6* or *R7* are introgressed into the potato genome does not rule out the possibility that these resistance factors could be allelic. The genetic studies conducted

so far in potato do not exclude any of these two possibilities (Mastenbroek 1953; Malcolmson and Black, 1966). The breeding history of the R3 containing cultivar Cardinal which was used as parent in the mapping population J92-6454 indicated that this resistance factor had originated from clone CPC2127 which also contained locus R7. The absence of R7 in this cultivar does not exclude linkage with R3. This rather favours the conclusion that both R3 and R7 were present in the accession of *S. demissum* at two homologous chromosomes in repulsion phase. The tester clones MaR6 and MaR7 could not be traced back to any common accession number. It was earlier reported that the resistance against race specific resistance in the host plant occur as a single locus, as multiple alleles or as a complex locus like the resistance to rust in flax, to powdery mildew in barley and to rust in maize indicated (reviewed by Pryor 1987). The resistance genes to downy mildew were clustered in four regions in Lettuce (Farrara et al., 1987) and also two complex resistance loci to *Cladosporium fulvum* were reported in tomato (Jones et al., 1993). The most direct way to find out either the relative distance or the allelism between R3, R6 and *R7* loci is to test for allelism. On the other hand, unequal crossing-over between these factors can lead to misinterpretation of the result. The phenomena of both unequal exchange and meiotic instability were observed in the *Rp1* locus conferring resistance to *Puccinia sorghi*, the causal agent for rust in maize (Sudupak et al., 1993). Regarding divergence in race specificity, a single gene in Arabidopsis confering resistance to two different *Pseudomonas syringae* strains was reported (Bisgrove et al., 1994). Also a model of two linked recessive genes was proposed to be responsible for controlling the resistance to southern leaf blight of maize (*Bipolaris (Helminthosporium) maydis*) (Chang and Peterson 1995). Also in potato, the inheritance pattern of resistance to potato virus Y (PVY) and potato virus X (PVX) suggested the involvement of two dominant genes (Vallejo et al., 1995). In the case of our results, more research is needed to answer the question whether allelism is involved or not.

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Chapter 4

Genetic localisation of transformation competence in diploid potato

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Abstract

In the course of improving diploid potato genotypes for transformation ability, selection for specific components affecting regeneration and transformation was carried out. From a segregating population between two good regenerating clones a selection was made to yield an optimal well-transforming and fertile genotype J92-6400-A16. This plant yielded predominantly diploid transformants and was heterozygous for the gene *R1*, conferring resistance to *Phytophthora infestans*. The speed of, and competence for, regeneration and transformation on both sides of the stem explant were improved. A competence factor for transformation was found to be linked with the *R1* locus and a molecular marker on chromosome 5. The male fertility of transformants was frequently decreased to a great extent, whereas Female fertility was not so markedly affected.

Introduction

Transformation of plants with *Agrobacterium tumefaciens* T-DNA is genotype- and protocol-dependent. Efforts have been made to improve transformation efficiency with modifications in culture technique, by interaction with specific genotypes (McCormick et al., 1986; Valvekens et al., 1988), or by the transfer of genetic factors from wild species (Koornneef et al., 1986). Transformation of heterologous plants with maize transposable elements is an extension of transposon-tagging technology to isolate genes in other species. While some plants like *Arabidopsis* are naturally well suited for the use of this technique (Aarts et al., 1993; Bancroft et al., 1993) other crop plants like potato are not. The tetraploid nature of the crop necessitates the use of diploid potato genotypes for genetic analysis and transposon tagging. For successful isolation of

genes in heterologous plants it is recognised that a relatively high tagging frequency of a specific locus is expected with linked transposons, as the maize elements transpose preferentially to linked locations (reviewed in Haring et al., 1991). This can be achieved by selecting for transposon-containing, T-DNA inserts located near to the target gene but requires the production of large numbers of transformants.

The maize elements transpose effectively in potato (Knapp et al., 1988; Frey et al., 1989; Pereira et al., 1991) but appropriate genotypes for employing tagging strategies have yet to be developed for the potato. This means that well-transforming diploid genotypes, preferably bearing the gene of interest, need to be selected. In potato, it is well known that a large variation for competence for regeneration (Taylor and Veillieux, 1992) and transformation exist and that both factors are highly protocol- and genotype-dependent (Visser, 1991). The effect of genotypes on regeneration has been analyzed in a number of plant species. The genetic factors involved can be dominant, as suggested in diploid alfalfa (Reisch and Bingham, 1980) and described and localised in tomato (Koornneef et al., 1987; 1993), or recessive, as found in the diploid cultivated potato *Solanum phureja* (Taylor and Veilleux, 1992).

Another problem related to the regeneration and transformation of diploid potato is tetraploidisation. For transposon-tagging research diploid transformants are required. For potato, it is generally known that tetraploidisation is genotype dependent. Competence for fast regeneration on stem explants is a key factor in avoiding this phenomenon (Visser, 1991).

In the present paper the selection of a genotype with good transformation ability and the discovery of linkage between transformation competence and the resistance locus *R1*, from the wild species *S. demissum*, is described. This plant provides the basis for tagging of the resistance locus *R1* to *P. infestans* by a *Ds*-element (Pereira et al., 1992).

Materials and methods

Plant material

R1r1 parent plants included the diploid (2n=2x=24) clone J91-6167-2, derived from a cv Hertha dihaploid cross "Her 64 X 87-1029-31", and clones J91-6146-(3, 4, 8, 9, 12, 15, 17, 19, 22 and 24), derived from a cv Saturna dihaploid cross "J90-6026-4 X 87-1029-31" (El-Kharbotly et al., 1994). The fertile *r1r1* diploid clone 87.1024-2 (Jacobsen et al., 1989) was used as the crossing parent because of its known

competence for regeneration and transformation (Pereira et al., 1992). These plants were grown in vitro as shoot cultures in 400-ml jars (four plants/jar) under 16-h light (3200 Lux) at 22-24°C on MS medium (Murashige and Skoog, 1962), supplemented with 30 g/l sucrose. They were also grown in a glasshouse and used as the female parent in a crossing program.

Regeneration from stem explants

Stem segments without the axillary buds from in vitro-grown shoots were used for the regeneration experiments. Forty or 80-120 explants per genotype were used in estimating the regeneration competence. They were placed on shoot-induction medium (MS with 8 g/l agar, supplemented with 30 g/l sucrose, 1 mg/l zeatin), 200 mg/l cefotaxime and 200 mg/l vancomycin. The plates were closed with cellophane tape and maintained under a 16-h photoperiod (3200 Lux) at 24°C for 56 days.

Transformation procedure

Transformation efficiency (TE) was determined with 37-43 and 19-40 explants per genotype from the 11 diploids and from the family J92-6400-A respectively. TE is the frequency of explants regenerating shoots after 10 weeks of growth on selective medium following treatment with *A. tumefaciens*.

The transformation was carried out according to Visser (1991) but with different media. The explants were pre-cultured for 1 day on M300 (Visser, 1991) covered with a piece of sterile Whatman no. 1 filter paper saturated with 2 ml of MS supplemented with 30 g/l sucrose, 1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 0.5 mg/l kinetin and 2 g/l casein hydrolysate without antibiotics. One-day later the explants were inoculated in a 2-day culture of *A. tumefaciens* for 15 min, blotted dry and placed back on the same medium. Two-days later the explants were transferred to a selection medium (shoot-induction medium; see regeneration), supplemented with 200 mg/l cefotaxime, 200 mg/l vancomycin and 100 mg/l kanamycin as a selectable antibiotic. The explants were sub-cultured on fresh medium 2 and 5 weeks after transformation and the Petri dishes were maintained under conditions for normal regeneration.

Bacterial strains

The *A. tumefaciens* strain AM8706 (Visser, 1989 a) was used. This strain contains the binary vector pBI121 (Jefferson et al., 1987) which has both the reporter gene β-glucuronidase (GUS) and the NPT II gene for kanamycin resistance.

In addition, the *A. tumefaciens* strain GV3101(pMP90RK) (Koncz and Schell, 1986), containing the recombinant binary vector pHPT::*Ds*-Kan (Pereira et al., 1992), was used for the production of *Ds*-containing transformants.

Determination of fertility

Female fertility of the selected clones was determined, after pollination with the fertile diploids J89-5040-2 and BE93-4002-3, in terms of berry set and seeds per berry. Male fertility was estimated after staining pollen with lactophenol acid fuchsin.

Screening for resistance

Preparation of the *P. infestans* inoculum and the inoculation procedure were carried out according to El-Kharbotly et al. (1994). Races 0 and 1 of *P. infestans* were kindly supplied by L.J. Turkensteen (Research Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands) and F. Govers (Department of Phytopathology, Wageningen Agricultural University , The Netherlands).

Ploidy level determination

The ploidy level of the transformants was estimated by counting the number of chloroplasts in the stomatal guard cells (Frandsen, 1967)

RFLP analysis

Parental clones (87-1024-2 , J91-6167-2) and their F_1 progeny were used for RFLP analysis. DNA extraction (Dellaporta et al., 1983), restriction digests, electrophoresis, blotting and hybridization procedures were all done as recommended by the suppliers. The RFLP probe GP21 (kindly supplied by Dr. C. Gebhardt, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany), linked to the *R1* locus (Leonards-Schippers et al., 1992) was used for hybridization of the Southern blots.

Results

Selection and testing of well-transforming R1r1 genotypes

The competence for regeneration and transformation was investigated in 11 diploid *R1r1* clones originating from the tetraploids cv Saturna and cv Hertha. The results, shown in Table 1, indicate that in this material there is a large variation for regeneration ability, which is the first step in the selection process for well-transforming

Table 1 Regeneration and transformation competence of 11 diploid clones.

Genotypes	Percentage of explants with shoots	
	Regeneration (31 days)	Transformation (70 days)
Originated from Saturna cv		
J91-6146-3	55 ± 7.9 a*	0 ± 0.0
J91-6146-4	88 ± 5.2 b	0 ± 0.0
J91-6146-8	75 ± 6.8 ab	0 ± 0.0
J91-6146-9	90 ± 4.7 b	0 ± 0.0
J91-6146-12	83 ± 6.0 ab	0 ± 0.0
J91-6146-15	75 ± 6.8 ab	0 ± 0.0
J91-6146-17	93 ± 4.2 b	3 ± 2.5
J91-6146-19	95 ± 3.4 b	0 ± 0.0
J91-6146-22	72 ± 7.1 ab	0 ± 0.0
J91-6146-24	78 ± 6.6 ab	0 ± 0.0
Originated from Hertha cv		
J91-6167-2	93 ± 4.2 b	24 ± 7.1

* There is no significant difference between percentages with the same letter at $P = 0.05$

genotypes. The percentage of regenerating explants varied between 55% and 95%. Screening for transformation showed only one genotype, J91-6167-2, derived from cv Hertha, with a high transformation efficiency (TE; 24%). J91-6167-2 appeared to be male sterile and berry and seed set were both relatively low, namely 0.2 berries/pollination and 21.2 seeds/berry. To overcome these fertility problems J91-6167-2 was crossed with clone 87.1024-2. The offspring plants of this cross (family J92-6400-A) were tested for regeneration, transformation efficiency, resistance to *P. infestans* and fertility traits.

Analysis of competence for stem-explant regeneration

Rapid regeneration is an important way of avoiding tetraploidisation. Therefore, regeneration of explants was scored after 18, 31 and 56 days (Table 2). Compared

Table 2 Regeneration competence of the progeny of 20 plants and their parents

Genotypes	Percentage of explants with shoots				
	Within			From two sides within	
	18 days	31 days	56 days	18 days	56 days
Parents					
87-1024-2	17 ± 3.8 a*	86 ± 3.5 a	99 ± 1.0 a	2 ± 1.4 a	18 ± 3.8 ab
J91-6167-2	15 ± 4.0 ab	88 ± 3.7 a	94 ± 2.7 ab	0 ± 0.0 a	34 ± 5.3 b
J92-6400-					
A1	4 ± 2.0 b	97 ± 1.7 a	98 ± 1.4 a	0 ± 0.0 a	12 ± 3.2 a
A2	2 ± 1.2	14 ± 3.2	32 ± 4.2	0 ± 0.0 a	0 ± 0.0
A3	18 ± 3.7 a	92 ± 2.7 a	99 ± 0.9 a	2 ± 1.3 a	11 ± 3.1 a
A4	5 ± 2.1 b	94 ± 2.3 a	99 ± 1.0 a	0 ± 0.0 a	63 ± 4.7
A5	18 ± 3.8 a	98 ± 1.4	96 ± 2.0 a	0 ± 0.0 a	22 ± 4.1 ab
A6	18 ± 3.8 a	91 ± 2.9 a	98 ± 1.4 a	3 ± 1.7 a	79 ± 4.1
A7	37 ± 4.8	95 ± 2.2 a	99 ± 1.0 a	4 ± 2.0 a	28 ± 4.5 ab
A8	1 ± 1.0	53 ± 5.0	81 ± 3.9 b	0 ± 0.0 a	3 ± 1.7
A9	4 ± 1.9 b	58 ± 4.9	86 ± 3.4 b	0 ± 0.0 a	0 ± 0.0
A10	60 ± 4.8	100 ± 0.0	100 ± 0.0 a	35 ± 4.6	92 ± 2.7
A11	3 ± 1.7	73 ± 5.0 a	99 ± 1.2 a	0 ± 0.0 a	5 ± 2.4 a
A12	19 ± 4.0 a	100 ± 0.0	100 ± 0.0 a	2 ± 1.4 a	57 ± 5.0
A13	14 ± 3.5 a	97 ± 1.7 a	99 ± 1.0 a	3 ± 1.7 a	59 ± 4.9
A14	25 ± 4.3 a	96 ± 2.0 a	97 ± 1.7 a	3 ± 1.7 a	82 ± 3.8
A15	30 ± 5.0 a	92 ± 3.1 a	98 ± 1.7 a	2 ± 1.7 a	63 ± 5.3
A16	41 ± 4.7	98 ± 1.3	100 ± 0.0 a	14 ± 3.3	93 ± 2.5
A17	42 ± 4.9	96 ± 1.9 a	98 ± 1.4 a	3 ± 1.7 a	67 ± 4.7
A18	0 ± 0.0	90 ± 3.0 a	91 ± 2.9 a	0 ± 0.0 a	13 ± 3.4 a
A19	39 ± 4.8	88 ± 3.2 a	94 ± 2.3 a	0 ± 0.0 a	0 ± 0.0
A20	51 ± 4.9	91 ± 2.8 a	100 ± 0.0 a	3 ± 1.6 a	33 ± 4.6 ab

* Significant difference from the parental clones at $P = 0.05$ is indicated by letters. The use of the same letter indicates no significant difference

with the parental clones, after 18 days, three groups of plants were distinguished in respect of the frequency of shooting explants: lower (four genotypes), no difference (ten) or higher (six). Thus, in six genotypes more stem explants were regenerating shoots than in the parental clones. After 31 days the frequency of regeneration was significantly increased for all genotypes but differences were still observed. Three genotypes were lower, 13 had no significant differences and four were higher than the parents. These differences disappeared after 56 days, except in the case of genotype J92-6400-A2 which invariably showed a relatively low frequency of regeneration.

In Table 2 the phenomenon of regeneration at both cut surfaces of the stem explants is also described. After 18 days, except J92-6400-A10 and J92-6400-A16, the parental clones and the sexual offspring had a low frequency of, or else no regeneration at both cut surfaces. Compared to the parental clones, scoring of this phenomenon after 56 days showed three groups namely "low" frequency (four genotypes), no difference (seven) and "high" frequency (nine) regeneration. The two best-performing genotypes were the same as those observed after 18 days (J92-6400-A10 and J92-6400-A16). Early regeneration was positively correlated with both early and late regeneration from both sides ($r = 0.65$ and $r = 0.54$, respectively). The fact that in most genotypes regeneration started at the original basal side of the explant, followed by regeneration at the apical side, suggests that regeneration on one side stimulates that on the other side. This was not the case in J92-6400-A10 and J92-6400-A16, with simultaneous regeneration at both sides, or in J92-6400-A2, J92-6400-A9 and J92-6400-A19, with regeneration only at one side.

Analysis of competence for plant transformation

The parents 87-1024-2 and J91-6167-2 and 26 sexual offspring plants (J92-6400-A) were investigated for TE. Besides resistance to race 0 of *P. infestans* and susceptibility to race 1, the segregation pattern of RFLP probe GP21, as well as the ability of regeneration under selective conditions after 18 days and from the two cut surfaces, were investigated (Table 3). In comparison with the TE of the parental clones differently performing progeny genotypes were found. After 18 days, no parental and four offspring plants with transformed shoots were observed. After 70 days, four genotypes (J92-6400-A8, J92-6400-A10, J92-6400-A11 and J92-6400-A13) had a lower frequency of explants with transformants than the best-performing parental genotype. The differences between several other genotypes and the parental clones were clear but not significant, due to the relatively low number of explants per genotype used. The phenomenon of transformation at two cut surfaces was not observed in the

Table 3 Competence for transformation, segregation for resistance to *Phytophthora infestans* race 0, and restriction polymorphisms with probe GP21 of a progeny of 26 plants and their parents

Genotypes	Transformation efficiency (%) within 70 days	Shoot induction within		RFLP[a] allele
		18 days	70 days from two sides	
Parents				
87-1024-2	15 ± 5.0 ab*	--	--	--
J91-6167-2	24 ± 6.6 a	--	--	+
Resistant J92-6400-				
A4	13 ± 6.8 a	--	--	+
A5	22 ± 8.0 a	--	+	+
A6	8 ± 5.2 a	--	--	+
A7	13 ± 6.2 a	+	+	+
A12	25 ± 7.7 a	+	+	+
A15	12 ± 6.5 a	--	+	+
A16	23 ± 8.3 a	+	+	+
A20	4 ± 3.9 a	--	--	+
A59	4 ± 3.8 a	--	--	+
A63	42 ± 9.7 a	--	--	+
A65	30 ± 8.0 a	--	+	+
A71	38 ± 9.9 a	--	+	+
A89	20 ± 7.3 a	--	--	+
A99	21 ± 8.3 a	--	--	+
Susceptible J92-6400-				
A1	12 ± 6.3 a	--	--	+
A2	5 ± 5.1 a	--	--	--
A8	0 ± 0.0 b	--	--	--
A10	0 ± 0.0 b	--	--	--
A11	0 ± 0.0 b	--	--	--
A13	3 ± 2.5 b	--	--	--
A17	0 ± 0.0 a	--	--	--
A18	0 ± 0.0 a	--	--	--
A19	9 ± 4.9 a	+	--	--
A50	30 ±10.2 a	--	+	+
A51	8 ± 5.4 a	--	--	--
A64	0 ± 0.0 a	--	--	--

+ = the phenomenon is present
-- = the phenomenon is absent
* The significant difference from the parental clones at $P = 0.05$ is indicated by different letters
a Marker GP21 linked to the R1 locus on chromosome 5

parental clones whereas it occurred in eight offspring genotypes. This is clearly different to what has been observed in the regeneration assay without transformation and selective growth (Table 2).

The offspring in Table 3 have been divided into resistant (*R1r1*) and susceptible (*r1r1*) genotypes. A significant positive correlation was found between TE and resistance (r = 0.57). This is a clear indication that, as a result of genetic linkage, an important part of the genetic variation connected with competence for transformation is associated with variation at the *R1* locus. To substantiate this observation all clones were investigated for polymorphisms with the probe GP21 which is closely linked to *R1* (Leonards-Schippers et al., 1992; El-Kharbotly et al., 1994). As expected, a positive correlation between GP21 and both *R1* and TE (r = 0.86 and r = 0.67 respectively) was observed. The magnitude of the correlations indicate that the order of the loci involved is *R1*--GP21--TE. The recombination percentage between *R1* and GP21 is 7.7%. The transformation competence of some susceptible clones (J92-6400-A2 and J92-6400-A51) are probably influenced by other genetic factors present in parental clone 87.1024-2.

For transposon-tagging purposes the resistant clones J92-6400-A16 and J92-6400-A12 appeared to combine most of the desired factors needed for the rapid production of a large number of transformants. J92-6400-A12 had the disadvantage of producing too many tetraploid regenerants (data not shown). Therefore J92-6400-A16 was selected for further research.

Fertility of J92-6400-A16 and its diploid transformants

The fertility of the parent J92-6400-A16 has been compared with that of the J91-6167-2 parent (Table 4). The pollen stainability, berry set per pollination, and seed set per berry of J92-6400-A16 were all much higher. The only disadvantage of J92-6400-A16 appeared to be late flowering.

Genotype J92-6400-A16 has been transformed with the *Ds*-element-containing *A. tumefaciens* strain. As expected, a high percentage of transformants (83%) proved to be diploid. A sample of diploid transformants with one or more T-DNA copies has been investigated for fertility traits. It turned out that pollen stainability was decreased to a level between 33.0% and 0.6%. Berry set per pollination from all transformants (except BET92-Ds-A16-18) was lower than that of the untransformed control J92-6400-A16 (Table 4), varying from 18.2 to 86.7%. The seed set per berry in all transformants was lower than in the control clone, varying from 18.9 to 77.4 seeds. These observations clearly indicate that both the male and female fertility of transformants are frequently affected to a certain degree.

Table 4 Male and female fertility of J91-6167-2, the selected clone J92-6400-A16, and 19 *Ds* transformants

Genotype	Male fertility (pollen stainability %)	Female fertility			
		Total no. of			
		Crosses	Berries	Seeds	Seeds/berry
J91-6167-2	0	41	10	212	21.2
J92-6400-A16	40	10	9	723	80.3
Single T-DNA copy					
BET92-Ds-A16 -5	8.9	30	25	1453	58.1
-7	22.9	9	3	88	29.3
-8	6.7	15	13	656	50.5
-100	25.0	27	18	736	40.9
-112	13.7	13	7	132	18.9
-234	5.9	19	8	424	53.0
-317	7.5	32	25	681	27.2
-379	33.0	11	2	128	64.0
-397	15.6	10	4	148	74.0
-409	8.0	23	18	636	35.3
-410	9.6	11	8	450	56.3
-416	7.0	11	8	269	33.6
-428	28.4	10	6	127	21.2
-438	14.5	57	38	1364	35.9
-465	0.6	50	37	1566	42.3
-473	6.7	33	20	1412	70.6
More than one T-DNA copy					
BET92-Ds-A16 -18	4.9	40	36	1197	33.3
-340	1.9	13	5	387	77.4
-453	16.5	40	24	869	36.2

Discussion

The correlations between the *R1* locus, the competence for transformation and the RFLP allele GP21 confirm the localization of the transformation-competence factor on chromosome 5. The number of genetic studies on transformation efficiency

described in the literature is low. The early study by Koornneef et al., (1986) showed the possibility of transferring the competence for transformation from *Lycopersicon peruvianum* to the cultivated tomato *L. esculentum*. Other studies have been mainly restricted to the detection of genotypic effects within a species, as recently described by Du et al., (1994) in the cultivated alfalfa. In potato, as in alfalfa (Du et al., 1994), competence for regeneration and transformation can be due to different factors. Genotypes like J92-6400-A10 and J92-6400-A17 (Tables 2 and 3) are early and well-regenerating but their competence for transformation is low, whereas genotypes such as J92-6400-A16 bear both competence factors. Variation in competence for transformation has earlier been described between tetraploid potato varieties (Hoekema et al., 1989).

Despite the good regeneration competence of cv Saturna (M. Goveia, personal communication) and its dihaploid progeny (unpublished data) a very low TE was found. This cultivar could only be transformed by an altered transformation protocol which has not been applied here. Our experiments showed that diploids, originating from this variety, were also recalcitrant for transformation. However a positive competence factor enabling transformation seemed to be linked to *R1* in the diploid *R1r1* clone J91-6167-2. This clone has been derived from the *R1*-bearing cultivar Hertha.

In the present study, genetic factors influencing shoot regeneration on stem explants appeared to be present but they were not investigated in detail. The outcome was that, in the sexual offspring, plants could be found with early and two-sided regeneration in a relatively high frequency of explants. In addition, the speed of regeneration differed between individual genotypes of the same progeny. The six genotypes which were faster than the parental clones in regeneration may be the result of the accumulation of genetic factors controlling this character. Indications of genetic variability in the speed of regeneration have been found earlier among diploids and monoploids derived from different diploid potato clones (M'Ribu and Veilleux, 1990).

The phenomenon of regeneration from two cut surfaces of the explant in diploid potato has earlier been described by Visser (1989 b). In the present study it is shown that this can be combined with high frequency competence for early regeneration and transformation. The advantage of this phenomenon is the induction of more independent transformants from the same number of explants, which save both labour and plant material.

Another important observation is that male fertility is decreased drastically in a relatively high frequency of transformants with one or more T-DNA inserts (Table

4). Female fertility is much less affected. This phenomenon was also reported earlier in diploid potato (Visser et al., 1989 c). For transposon-tagging research, female fertility must be relatively high when high numbers of seeds are needed in screens for the "insertion mutant" among sexual offspring.

It can be concluded that variation is available for both regeneration and transformation competence. These competence factors could easily be selected in combination with the well-segregating resistance factor *R1* and with sufficient male and female fertility in the diploid clone J92-6400-A16.

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Chapter 5

Localization of *Ds*-transposon-containing T-DNA inserts in the diploid transgenic potato: Linkage to the *R1* resistance gene against *Phytophthora infestans* (Mont.) de Bary.

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Abstract

The *Dissociation* transposable element (*Ds*) of maize containing NPTIIgene was introduced into the diploid potato (*Solanum tuberosum*) clone J91-6400-A16 through *Agrobacterium tumefaciens* mediated transformation. Genomic DNA sequences flanking the T-DNAs from 187 transformants were obtained with Inverse Polymerase Chain Reaction (IPCR) or Plasmid Rescue (PR) techniques and used as probes for RFLP linkage analysis. The RFLP map location of 60 T-DNAs carrying *Ds*-Kan was determined. The T-DNA distribution per chromosome and the relative distance between the T-DNAs appeared to be random. All the 12 chromosomes have been covered with *Ds* containing T-DNAs potentially enabling tagging of any gene in the potato genome. The T-DNA insertions of two transformants, BET92-Ds-A16-259 and BET92-Ds-A16-416, were linked in repulsion to the position of the resistance gene *R1* against *Phytophthora infestans*. After crossing of BET92-Ds-A16-416 with a susceptible parent, 4 desired recombinants (*Ds*-carrying T-DNA linked in coupling phase with *R1*-gene) were discovered. These will be used for tagging the *R1* gene. The efficiency of the pathway from the introduction to localization of T-DNAs is discussed.

Introduction

The vertical resistance to late blight(*Phytophthora infestans* (Mont.) de Bary) in potato (*Solanum tuberosum* L. 2n=4x=48) is controlled by major dominant *R* genes (Mastenbroek 1953; Malcolmson and Black 1966). Although *R* genes did not provide

durable resistance (Turkensteen 1993), their molecular isolation is important for a better understanding of this specific plant defense mechanism. The products of *R*-genes are unknown and this hampers the usual method of gene isolation via protein characterization and cDNA cloning. An alternative approach can be through insertional mutagenesis (transposon tagging). Endogenous transposons in maize and *Anthirrhinum* have been used for transposon tagging of genes whose products are not known but have an easily distinguishable mutant phenotype (Walbot 1992). Several studies have demonstrated that the maize transposable element *Activator (Ac)* is capable of transposition in heterologous hosts including several members of the Solanaceae such as tobacco, tomato and potato (Baker et al., 1986; Knapp et al., 1988; Yoder 1990). Recently successful tagging and cloning of various genes using maize transposable elements in heterologous systems have been reported (Aarts et al., 1993; Chuck et al., 1993; Jones et al., 1994).

The *Ac* element showed a tendency to transpose to closely linked positions on the same chromosome whether it is in maize (Greenblatt 1984; Dooner and Belachew 1989) or in heterologous hosts like tobacco (Jones et al., 1990; Dooner et al., 1991) and *Arabidopsis* (Keller et al., 1993). In tomato, clusters of transposed *Ac* elements (Osborne et al., 1991; Belzile and Yoder 1992) and *Ds* elements (Thomas et al., 1994), either linked or unlinked to the donor locus, have been observed. Studying the reinsertions of the *Ds* element from two closely linked loci Knapp et al., (1994) showed that in one family the transposed *Ds* element preferentially reinserted close to the T-DNA, but not in the other family. The short-range transposition of maize transposons on the same chromosome was successfully used to tag the maize *R-nj* locus (Dellaporta et al., 1988) and recently the *Cf-9* gene of tomato (Jones et al., 1994).

Two of the main prerequisites for efficient transposon tagging in potato are: (1) knowledge of the RFLP map position of the target gene, (2) establishing diploid transformants carrying *Ds* closely linked to the target gene. *R1* and *R3*, conferring resistance to *P. infestans* in potato, were mapped to chromosome 5 and 11 respectively (Leonards-Schippers et al., 1992; El-Kharbotly et al., 1994), and recently both *R6* and *R7* also on chromosome 11 (El-Kharbotly in preparation). Here we report on diploid potato clones containing *Ds* elements-carrying T-DNAs at defined genomic positions and their chromosomal distribution with emphasis on linkage to the *R1* resistance locus.

Materials and methods

Plant material and transformation

Transformation of the diploid potato (2n=2x=24) (*Solanum tuberosum*) clone J92-6400-A16 was carried out with *Agrobacterium tumefaciens* as described by El-Kharbotly et al. (1995). Selected transformants were used as crossing parent with the diploid clone BE93-4002-3. Clone BE93-4002-3 is a hybrid between J92-6222-24, originating from G254 X G609, (Hermsen, 1978) and J92-6400-A16. Both diploid clones J92-6400-A16 and BE93-4002-3 are heterozygous for the resistance gene *R1* against *Phytophthora infestans*. Also the fertile *r1r1* diploid clone J89-5040-2 (El-Kharbotly et al., 1994) was used as male parent in a test cross.

Bacterial strains

The *A. tumefaciens* strain GV3101(pMP90RK) (Koncz and Schell, 1986) containing the recombinant binary vector pHPT::*Ds*-Kan (Pereira et al., 1992) was used for the production of *Ds*-containing transformants. Selection of transformants was based on kanamycin resistance as the selectable marker. Hygromycin resistance was used to monitor excision of the *Ds* element.

To study excision of the *Ds* element, the selected transformants were retransformed with *Ac* transposase gene. In the CaMV35S-*Ac*-methotrexate resistance construct both the *Ac* transposase and methotrexate resistance genes are controlled by the CaMV35S promoter for constitutive expression. Either methotrexate or hygromycin resistance was used as a selectable marker for the transformation. The regenerated shoots were tested for rooting on 0.15 mg/l methotrexate or 40-50 mg/l hygromycin.

Screening for resistance

Preparation of *P. infestans* inoculum (races 0 and 1) and the inoculation procedure was carried out according to El-Kharbotly et al. (1994). The races 0 and 1 were kindly supplied by Dr. L.J. Turkensteen (Research Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands) and Dr. F. Govers (Department of Phytopathology, Wageningen Agricultural University , The Netherlands).

Ploidy level determination

Ploidy level of the transformants was determined by counting the number of chloroplasts in the stomatal guard cells (Frandsen 1967)

Molecular analysis

Potato genomic DNA sequences flanking the integration site of the *Ds* element containing T-DNA were isolated by either IPCR (Triglia et al., 1988) or via plasmid rescue by electroporation (Dower et al., 1988). They were used for RFLP analysis as described by Pereira et al., (1992). DNA extraction (Dellaporta et al., 1983), restriction digestion, electrophoresis, blotting and hybridization procedures were done as recommended by suppliers. More details on RFLP markers, isozymes, genetic markers, linkage analysis and map construction are provided else where (Jacobs et al., 1995).

Cytogenetic analysis

The physical length of each chromosome as well as the euchromatin contents were estimated using photographs of pachytene chromosomes (5-10 per chromosome) of all 12 pairs of diploid potato. Chromosomes were selected from mid-pachytene stage in order to avoid the over or under estimation of the physical lengths. Identification of potato chromosomes was done according to Ramanna and Wagenvoort (1976) which was based on the system of tomato chromosome classification. This classification was concurrent with the RFLP maps of the diploid potato as well as that of the tomato genome.

Statistical analysis

Based on the physical or RFLP map length, the number of integrated T-DNAs per chromosome was tested for random distribution. Because of the low numbers, X^2 was calculated using the Yate's correction. $X^2=\Sigma[\ |\ O-E\ |\ -0.5]^2/E$, where O and E are the observed and the expected number of T-DNAs integrated per chromosome, respectively.

In addition, the clustering versus random integration was examined using Kolmogorov-Smirnov test (Kolmogorov 1980). The expected cumulative frequency distribution of the integrated T-DNAs were calculated based on the potato CE "join map" (Jacobs et al., 1995). Chromosome 5 and 10 were excluded from the calculation because only the separate male and female map is available (see Jacobs et al., 1995). The absolute differences between the observed and the expected cumulative frequencies were compared with the critical value of the Kolmogorov-Smirnov function at 0.05 significance level.

Results:

Generation of the transformants

Potato transformants were generated from 400 stem explants which were inoculated with *A. tumefaciens*. Because of double sided regeneration of explants, 491 independent regenerants were isolated, 377 of them rooted on MS medium containing kanamycin (Fig. 1). These plantlets were brought to the greenhouse and the ploidy level was determined. Chloroplast counts showed 65 tetraploid genotypes. These tetraploid plants were discarded and the remaining 312 diploid transformants were used for further analysis. All diploid transformants showed, as expected, resistance to *P. infestans* race 0 and susceptibility to race 1. Two transformants showed

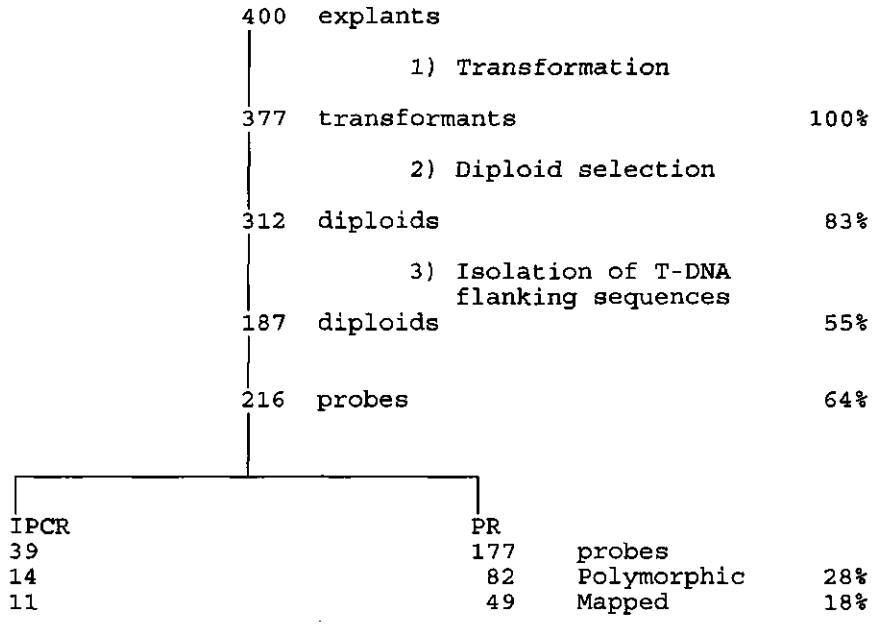

Fig. 1 The efficiency of steps from transformation of diploid potato to localization of the T-DNA inserts by flanking genomic DNA probes.

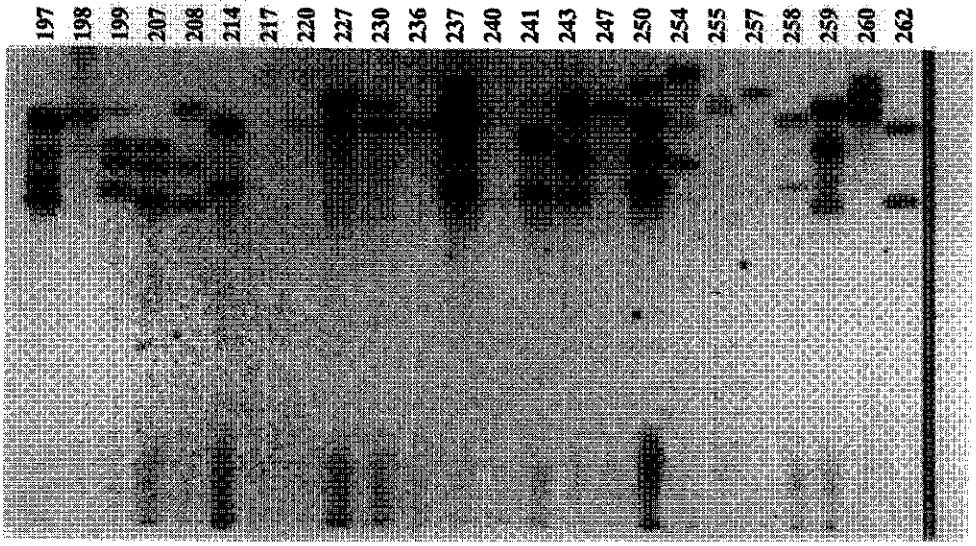

Fig. 2 The T-DNA copy number in diploid transgenic potato clones, as determined by the number of *HindIII* fragments hybridizing to a left border probe of the T-DNA. The code of the transformants is BET92-Ds-A16-(1 to 491). For simplicity, only the last three digits are written. Transformant numbers BET92-Ds-A16-217 and 240 had deletion at the left border, numbers BET92-Ds-A16-198, -220, -236 and -257 had a single copy number. All other transformants, which are shown here, had more than one copy of T-DNA.

variegation (yellow leaves) and one of them died before sampling for molecular analysis.

Characterization of Ds-Kan-bearing transformants

The diploid transformants containing pHPT::*Ds*-Kan exhibited kanamycin resistance but were hygromycin susceptible because the *Ds*-Kan was inserted in the untranslated leader of the hygromycin resistance gene (Pereira et al., 1992). The mobilization of *Ds* can thus be monitored by selection for hygromycin resistance.

The copy number and the structural integrity of the introduced T-DNA in transformants were analyzed by Southern hybridization. The T-DNA copy number, as determined by the number of Hind III fragments hybridizing to a left border probe of the T-DNA (Fig. 2), appeared to be single copy in 24% of the transformants while

2-8 copies were present in the rest. Structural integrity of introduced T-DNAs was examined by rehybridization using a probe from the right border. Further analysis was done in plasmid rescued T-DNA structure. With few exceptions (of left border deletions), no grossly rearranged T-DNA structures were observed.

Isolation of T-DNA flanking sequences

Genomic DNA sequences flanking the right or left border of T-DNA insertions were isolated by IPCR or plasmid rescue (PR). They were subsequently used as probes in RFLP linkage analysis in order to map the corresponding T-DNA insertion. So far 216 (PR/IPCR) probes were successfully isolated from 187 individual transformants out of 312 transformants attempted (Table 1, Fig. 1). Based on the experimental conditions outlined before, plasmid rescue (PR) products of up to 10 kb were generated. Seven clones were larger than 3 kb size (table 1). All cloned flanking DNA fragments were digested either with restriction enzymes *HindIII-BglII* or with a combination of *EcoR1, Xba1, Xho1, EcoRV* and *HindIII + BglII* in order to increase the probability of generating smaller probes (<3 kb) suitable for hybridization.

Table 1 Molecular analysis of the T-DNA flanking probes in diploid transgenic potato.

Size	Signal			# of probes total	Poly-morph	Mapped from	
	Single	Multiple	No			Single	Multiple
nd*	3	1	11	15	2	0	0
<300	0	0	2	2	0	0	0
300-500	7	7	5	19	4	2	1
-1000	58	24	15	97	46	28	4
-1500	28	11	8	47	29	15	4
-2000	6	5	2	13	8	2	1
-2500	9	1	1	11	7	3	0
-3000	0	2	3	5	0	0	0
>3000	0	0	7	7	0	0	0
TOTAL	111	51	54	216	96	50	10

Mapping of T-DNA insertions

Labelled IPCR or PR derived DNA sequences were used to probe Southern blots of CE, a segregating diploid potato population, (Jongedijk and Ramanna 1989). Genomic

DNA was digested with *EcoR1, EcoRV, HindIII* and *BglII* to detect suitable RFLPs for molecular mapping. The majority of T-DNA flanking sequences (111) hybridized to a single copy sequence (Table 1). Fifty one IPCR/PR clones gave a hybridization pattern characteristic for a multigene family or dispersed repeat sequences. Ninety six probes showed polymorphisms with either a single band (83) or multiple bands (13). In total 60 probes were mapped (Fig. 3). The probes that showed polymorphisms had a size range from 500-2500 bp while the 500-1500 bp probes were the most efficient for mapping (Table 1). The percentages of mapped probes were 60% and 77% from those that showed single and multiple polymorphic bands respectively. Though the polymorphism was much higher for the probes which gave a single band than for those that gave multiple bands, the mapping efficiency from these polymorphic probes was higher in case of multiple bands. A disadvantage of probes giving multiple bands is the possibility of mapping one T-DNA at different locations. An example of such a case was the flanking T-DNA probe TDs-436 (genotype BET92-Ds-A16-436) which was mapped to chromosomes 2, 4 and 5. In such cases the exact location could be determined by genetic segregation analysis. Based on the total number of investigated probes, probes with a single polymorphism were mapped more efficiently than those with multiple polymorphisms (45% versus 20%) (Table 1).

T-DNAs of both BET92-Ds-A16-259 and BET92-Ds-A16-416 were localized on chromosome 5 and linked to the R1 locus conferring resistance to *P. infestans*. Further analysis showed that the linkage was in repulsion phase at 13 and 25 cM respectively.

Chromosomal Distribution of T-DNAs

T-DNA insertions were identified on all chromosomes. The expected number of T-DNAs per chromosome was calculated according to chromosome length or euchromatin content assuming no chromosomal preference for T-DNA integration (Table 2). The genotype BET92-Ds-A16-436 was excluded from the analysis because the flanking sequence of the T-DNA was mapped to three different chromosomes. To simplify the analysis, two observed classes were created for each chromosome. These consisted of T-DNA insertions that were either located on that particular chromosome or not, enabling a simple X^2-test with one degree of freedom to be performed for each chromosome. No apparent biases (at $P=0.05$) were observed in this test when the expected number of T-DNAs per chromosome was calculated according to chromosome length or to euchromatin content. The random distribution was also observed when the same X^2-test was applied to the data according to the chromosome length based on the RFLP map of the CE population (Jacobs et al., 1995). Integration

Table 2 Statistical analysis of the chromosomal distribution of T-DNAs in diploid transgenic potato.

Chromosome			Observed number	Based on chromosome length			Based on euchromatin content		
number	length μm	Euchromatin content μm	of T-DNAs	Expected number T-DNAs	X^2	P	Expected number T-DNAs	X^2	P
1	71.90	54.04	3	7.59	2.53	>0.10	8.01	2.94	>0.05
2	50.12	39.00	7	5.29	0.30	>0.50	5.78	0.10	=0.75
3	33.50	22.50	6	3.54	1.16	>0.25	3.33	1.49	>0.25
4	49.10	39.20	5	5.18	0.02	=0.90	5.81	0.02	=0.90
5	47.20	31.01	2	4.98	1.35	>0.15	4.60	1.04	>0.25
6	47.70	37.11	5	5.04	0.05	>0.75	5.50	0.00	>0.99
7	37.43	24.90	4	3.95	0.06	>0.75	3.69	0.01	>0.75
8	46.30	34.80	7	4.89	0.58	>0.25	5.16	0.38	>0.50
9	46.70	34.14	4	4.93	0.04	>0.75	5.06	0.07	>0.75
10	39.80	22.11	6	4.20	0.43	>0.50	3.28	1.60	>0.10
11	42.50	28.90	3	4.49	0.24	>0.50	4.28	0.15	>0.50
12	46.50	30.41	7	4.91	0.56	>0.25	4.51	0.95	>0.25

NOTE: X^2 analysis of the chromosome distribution of 59 T-DNAs. X^2 values were calculated using Yate's correction for whole numbered observations i.e. $X^2=\Sigma[\ |\ O-E\ |\ -0.5]^2/E$.

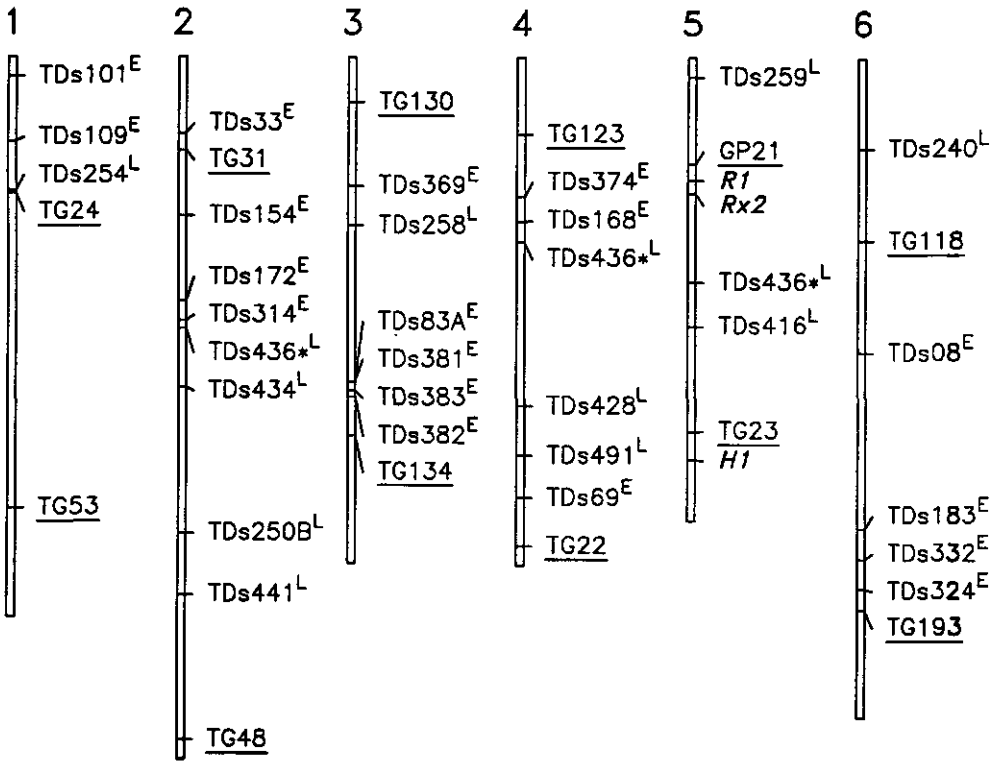

Fig. 3 RFLP "join map" of (CE) diploid potato population showing the distribution of *Ds* element containing T-DNAs on chromosomes 1-12. Chromosomes 5 (E) and 10 (C) are presented in this diagram
because only the separate male (E) and female (C) map is available. Resistance genes (in italic) against potato pathogens were incorporated from different maps at approximate positions.

* = The flanking sequence of the T-DNA of the genotype BET92-Ds-A16-436 was mapped to three different locations.
E = Early regenerated transformant.
L = Late regenerated transformant.

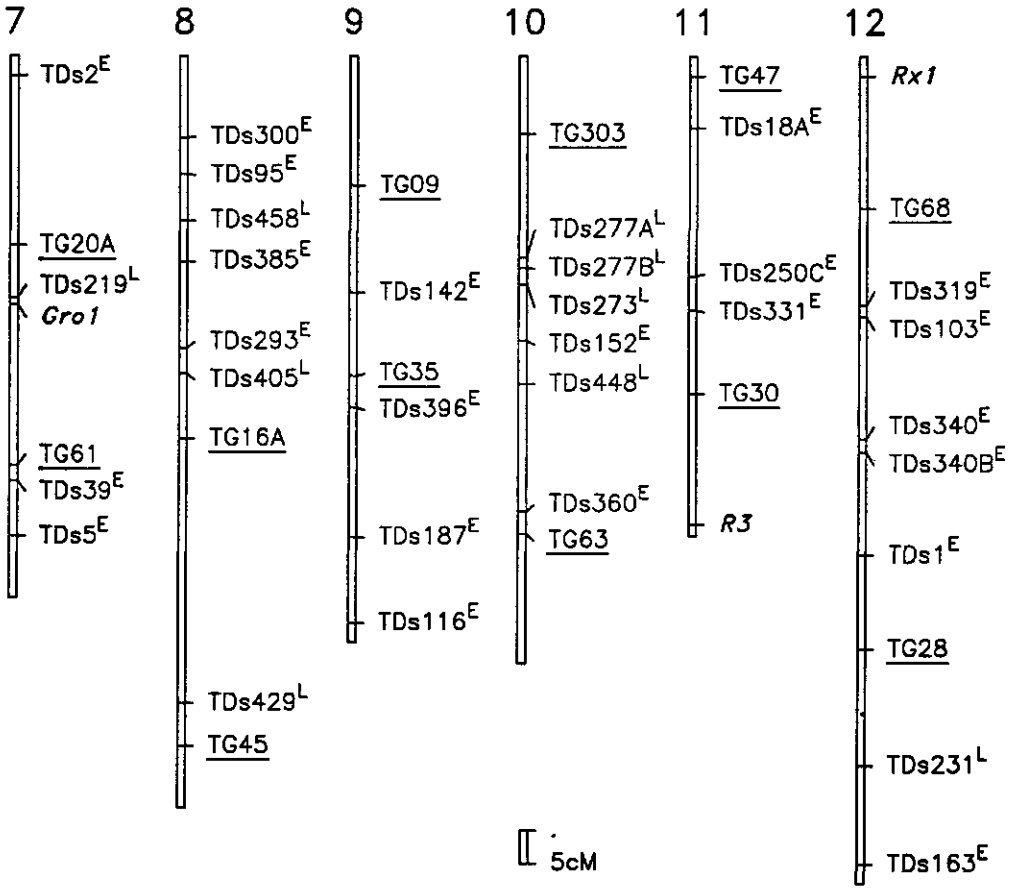

Figure 3, continued

of the T-DNAs seemed, in many cases, to be in clusters (Fig 3). However, statistical analysis using Kolmogorov-Smirnov test (Kolmogorov 1980) showed no difference with the normal distribution (Fig 4). It was also noticed that the T-DNAs (two to four) from independent, early or late regenerated, transformants were integrated at particular locations close to each other (1-6 cM). The linked T-DNAs of early regenerated transformants mapped on chromosomes 3, 4, 6, 7, 8 and 12 while those from late regenerated transformants on chromosome 10 (Fig 3). All the linked or unlinked T-DNAs which were integrated on chromosome 5 (three T-DNAs) and on chromosome 9 (four T-DNAs) were from late and early regenerated transformants respectively. The mapping efficiency of early and late regenerated transformants was almost the same (22% and 19% respectively) and statistical analysis was not performed because of the relatively low number of mapped T-DNAs per chromosome.

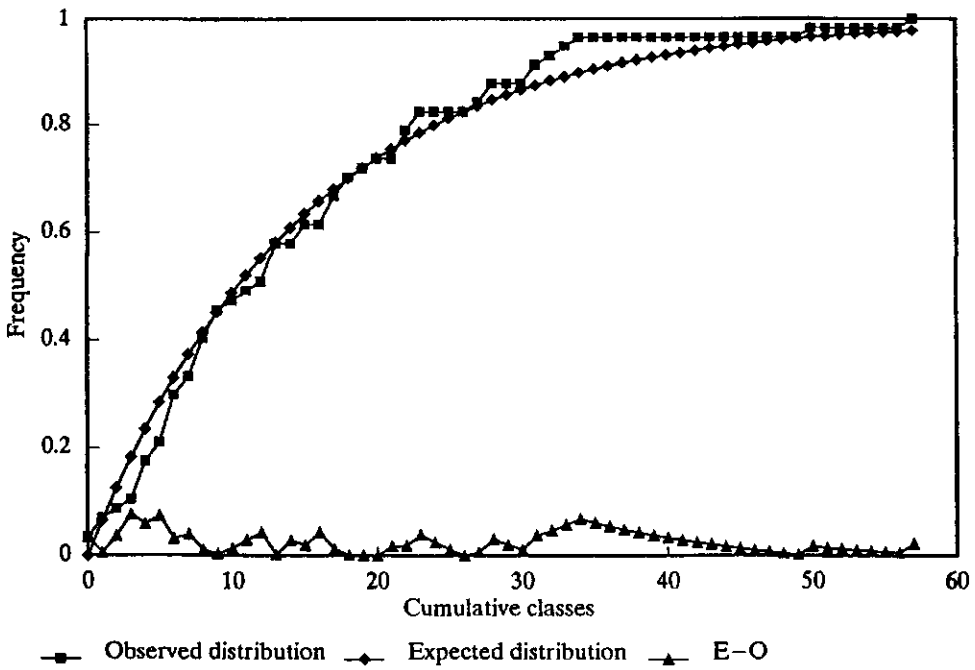

Fig. 4 Cumulative distribution of the integrated T-DNAs according to CE "join map" (Chromosome 5 and 10 were excluded from the calculation).
Kolmogorov-Smirnov critical value=0.17669 at $P>0.05$. The absolute difference between observed and expected distribution is less than the critical value. This means that the T-DNAs were integrated randomly.

Inheritance of the P. infestans resistance and T-DNAs in F_1 progenies

Fifteen transformants with a single copy T-DNA each and three with more than one copy were randomly selected and crossed with BE93-4002-3 which was resistant to *P. infestans* (R1r1) but susceptible to kanamycin. The T-DNA transmission was scored by selection for kanamycin resistance in the progenies. Seeds (25 per cross combination) were sown *in vitro* and cuttings of each seedling were tested for rooting on kanamycin containing medium and later transplanted into a greenhouse to test for resistance to *P. infestans* race 0. All F*1* progenies, except the progeny of BET92-Ds-A16-409, showed the expected Mendelian segregation ratios for resistance to *P. infestans* (3:1) and kanamycin (1:1). The F*1* progeny of BET92-Ds-A16-409 segregated 16:5 ($X^2_{1:1}$ = 5.76, $0.05 > P > 0.025$) and 10:11 ($X^2_{3:1}$ = 8.4, $0.01 > P > 0.005$) resistant versus susceptible for kanamycin and *P. infestans* respectively. This indicated distorted segregation for both loci. Linkage was observed between the *R1* and T-DNA locus in the F*1* progeny of BET92-Ds-A16-416. This clone, containing a single copy of T-DNA, was also crossed with the *r1r1* susceptible diploid clone J89-5040-2. Screening of the F*1* progeny for both kanamycin and *P. infestans* resistance confirmed the genetic linkage. Four phenotypes were observed; a) resistant to both *P. infestans* and kanamycin (four genotypes), b) resistant to *P. infestans*, susceptible to kanamycin (12), C) susceptible to *P. infestans*, resistant to kanamycin (21) and d) susceptible to both *P. infestans* and kanamycin (two). This segregation pattern showed linkage between *R1* and the T-DNA insert of BET92-Ds-A16-416 in repulsion phase (18 cM). Four recombinant clones were identified with linkage of both characters in the coupling phase and they are used as selected material in further tagging experiments of the *R1* gene.

Excision of Ds-Kan

To investigate the excision of an inserted *Ds*-element, four transformants with a single T-DNA copy were selected for retransformation with *Ac* transposase gene. Before retransformation, the response of these plants on hygromycin containing medium was tested critically. They clearly showed different levels of susceptibility to this antibiotic varying between 20-40 mg/l (Table 3). Stem explants from these transformants were retransformed with the CaMV35S-*Ac* containing T-DNA construct and placed on a shoot regeneration medium containing either methotrexate (transformation marker) or hygromycin (excision marker). The untransformed clone J92-6400-A16 was used as a control. The difference in behaviour on the two selection media was observed to be significant only in the genotype BET92-Ds-A16-112 (Table 3). The low performance of this genotype on methotrexate containing medium might be connected with the

Table 3 The level of hygromycin resistance and the reaction after retransformation with Ac-Mtx of four diploid potato transformants containing HPT::*Ds*-Kan.

Code	Hygromycin selective concentration (mg/l)	Percentage of explants with transformants		
		Methotrexate		Hygromycin
BET92-Ds-A16-				
5	>20	24 ± 6.0	*a A**	13 ± 4.1 *A*
7	>30	0 ± 0.0	*B*	2 ± 1.8 *B*
100	>40	17 ± 7.0	*a C*	6 ± 3.5 *C*
112	>30	0 ± 0.0	*D*	13 ± 5.2 *F*
Control				
J92-6400-A16	>20	22 ± 8.6	*a G*	0 ± 0.0 *H*

* Percentages indicated by the same letter (within transformants are small letters and between selection are capital letters) have no significant difference.

relatively high toxicity level of this antibiotic on stem explants. The genotype BET92-Ds-A16-7 showed low performance on both media. However, the other two genotypes BET92-Ds-A16-5 and -100 produced more transformants on methotrexate containing medium than on hygromycin. Southern hybridization with probes of the CaMV35S-*Ac* construct confirmed the presence of *Ac* in all regenerated plants (data not shown). It was also observed that all plants, regenerated on medium containing methotrexate, were hygromycin resistant and vice versa. These observations suggest that the excision of *Ds* took place shortly after transformation. Using only hygromycin resistance during transformation as selectable agent instead of methotrexate had the advantages of saving both time and labour in order to obtain transformants with an excised *Ds* element.

Discussion:

We have established 57 transgenic clones carrying *Ds* at 60 known positions on the potato RFLP map (Jacobs et al., 1995) with the goal of using them for targeted transposon tagging experiments. The T-DNA insertions are distributed randomly all over the genome (Table 2) and can serve as starting-points for transposon tagging experiments by transposon induced mutations of target genes like *Gro1* and *H1* for nematode *(Globodera rostochiensis)* resistance on chromosome 7 and 5 respectively (Barone et al., 1990; Gebhardt et al., 1993; Pineda et al., 1993), *Rx1* and *Rx2* for

potato virus X (PVX) resistance on chromosome 12 and 5 respectively (Ritter et al., 1991), *R1* and *R3* for fungus *(Phytophthora infestans)* resistance on chromosome 5 and 11 respectively (Leonards-Schippers et al., 1992 and El-Kharbotly et al., 1994) or other genes of interest. We have indicated the efficiency of each step from transformation up to mapping of integrated T-DNAs. Starting with 377 transformants of which 312 were diploid, T-DNA inserts of only 57 plants could be localised. This information is helpful to estimate in advance the time and costs needed to select for a T-DNA linked to a particular gene of interest.

Transposon tagging strategies utilising *Ds* are potentially advantageous over strategies using *Ac*, since the transposon and transposase source are separated, enabling selection of stably expressed transposon-induced mutations. The introduced *Ds*-containing construct was equipped with two selectable markers. The neomycin phosphotransferase gene (NPTII) inserted inside the *Ds* element allowed the selection of plants harbouring the *Ds*-Kan element. The hygromycin phosphotransferase gene (HPT) was used to detect the excision of the element from its donor site in the T-DNA. After retransformation with a transposase gene, this marker was used to select for plants that had undergone an excision event. We could demonstrate that the hygromycin resistance marker gene may show some background activity even in the absence of excision (Table 3). This may be related to the position of the integrated T-DNA in potato genome. In tomato, in which a similar construct was introduced, molecular analysis indicated that read-out transcriptional activity associated with the end of the *Ds* element was responsible for expression of the HPT gene (Rudenko et al., 1994) and efficient selection for excision could only be obtained by increasing the hygromycin level (Overduin 1994). This was also observed in our tested genotypes.

In this study a detailed analysis of T-DNA integration in the genome of diploid potato was made. Earlier studies in tomato, *Petunia hybrida* and *Crepis capillaris* on a smaller number of transformants, revealed also no obvious chromosomal bias for T-DNA integration (Ambros et al., 1986; Chyi et al., 1986; Wallroth et al., 1986). Recently, random T-DNA integration of an octopine-metabolising *Agrobacterium* strain was recorded in tomato transformants (Thomas et al., 1994). In our statistical analysis (Table 3) the expected number of T-DNA insertions was calculated according to euchromatin content, total chromosome physical length or on the basis of the RFLP map length. We consider the euchromatin content to be more appropriate than total chromosome length because studies in which T-DNA genes are activated by plant genes suggested that T-DNA integrates preferentially into transcribed loci (Koncz et al., 1989; Herman et al., 1990).

Cytological and deletion analyses of the tomato genome (Khush and Rick 1968)

suggested that genes are largely absent from heterochromatin and we consider it to be a less likely target for T-DNA integration. Although our data did not reveal a significant chromosomal bias (Table 2), the sample size considered here may still not be sufficiently large to reveal possible chromosomal preferences for T-DNA integration. Also our data suggested no clustering in the integration of the independently inserted T-DNAs.

The early or late regeneration of transformants carrying T-DNAs at certain places of particular chromosomes may be due to increase or decrease of the levels of regeneration competence as a result of position effects. This phenomenon could be relevant for selecting more effectively transformants containing T-DNAs on particular chromosomes.

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Chapter 6

Race specific resistance against *Phytophthora infestans* in potato is controlled by more genetic factors than only *R*-genes.

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Abstract

For RFLP mapping of *R*-genes, determining resistance to specific races of *Phytophthora infestans* in tetraploid potato, it is necessary to develop well segregating populations at the 2x level. During mapping studies evidence was obtained that more genetic factor(s) are involved in the expression of *R*-genes. Two experiments are described in which such an additional genetic factor was suppressing or enhancing the expression of unknown R_n and R_i factors. R_n and R_i appeared to be present in the investigated plant material containing *R4*, *R10* or in one of the susceptible crossing parents. In a third experiment the expression and the segregation of the well known *R1* gene was influenced by an additional genetic factor. In that case there were indications for a dominant suppressor. This was established by the selection of susceptible plants carrying a RFLP allele of probe *GP21* closely linked to *R1*. In three of the four F_1 populations resulting from crosses between such susceptible plants and susceptible tester plants, resistant progeny were found. The resistance appeared to be *R1*-specific. This clearly indicates that in three of the four investigated susceptible plants, the *R1* gene was still present but not expressed.

Introduction

Phytophthora infestans (Mont.) de Bary is the causal agent of late blight in potato (*Solanum tuberosum*) which induces severe reduction in tuber yield. In the early breeding programmes resistance genes (*R*-genes) were introduced from the hexaploid wild species *S. demissum*. So far 11 *R*-genes have been identified (Black et al., 1953; Malcolmson and Black, 1966; Ross, 1986; Wastie, 1991). The genetic studies (Mastenbroek, 1953; Malcolmson and Black, 1966) on the *R*-genes showed simple Mendelian inheritance of this dominant trait. On the other hand, deviant segregation for *R*-loci from expected ratios was found by many researchers. Black (1945) found that segregation of resistant and susceptible plants in the progenies, of triple and

multiple species hybrids, showed an excess of recessive susceptible individuals compared with the expected standard ratios of 1:1, 3:1 and 15:1. Mastenbroek (1953) also reported that in some crosses, there was a deficit of immune seedlings. Moreover, excessive amounts of susceptible plants were reported in backcrosses with susceptible plants and selfings of *R2*, *R4*, *R6*-containing potato plants (McKee, 1961). Recent inheritance studies in diploid potato clones containing *R*-genes (El-Kharbotly et al., 1994) revealed an excess of susceptible genotypes and also an altered race specificity. Additionally, segregation analysis of resistance in potato (Colon et al., 1995) suggested the presence of a second genetic factor affecting the partial resistance against *P.infestans*.

Genetic factors other than *R*-genes which are required for the expression of race specific resistance have been identified in crops like tomato and barley (Hammond-Kosack et al., 1994; Freialdenhoven et al., 1994). Also a suppressor gene that inhibits the resistance to stem rust in wheat has been localized (Kerber, 1991). In wheat, a rust resistance factor introduced from rye showed dominant or recessive inheritance (in different genetic backgrounds). This is probably another explanation for the irregular patterns of inheritance (Bartos, 1993).

Here, we have investigated the inheritance of resistance patterns in several offspring of specific genotypes expressing a particular *R*-gene using a set of races of *P. infestans*. Altered resistance patterns were found indicating the presence of hidden *R*-genes. Their expression could be explained by the segregation of a second factor influencing expression of these *R*-genes. The use of RFLP analysis allowed the detection of the presence of such a second factor influencing expression of the *R1* gene.

Materials and methods

Plant material

The tetraploid potato *(Solanum tuberosum)* clone PI 203900 (coded J90-6028), containing R4 (Spielman et al., 1990) and cultivar Primeri, containing *R10*, (Dutch variety list; Anonymous 1991) were used as parental plants for dihaploid induction. Dihaploids (2n=2x=24) were induced through prickle pollination using the diploid species, *S.phureja*, clone IVP 101 (Hutten et al., 1994). The dihaploids obtained were tested for resistance with *Phytophthora infestans* race 0. The resistant dihaploids were grafted onto tomato root stock (cv. Virosa) for flower induction and pollination. The diploid clones IVP 48 (Hermsen and Verdenius 1973), 87-1024-2 (Jacobsen et al., 1989), J89-5040-2 (El-Kharbotly et al., 1994) and SUH3711 were used as pollinators.

They were fertile and susceptible to *P. infestans (rr)*.

The amylose-free and susceptible clone J90-600-4 (Flipse et al., 1995) was used as tetraploid crossing parent. Family J92-6400-A (El-Kharbotly et al., 1995) was segregating for the *R1* gene. Susceptible clones of this family that still contained the RFLP allele *GP21*, which was closely linked to the *R1* gene, were selected. These were used in crosses with the susceptible clone SUH3711.

Screening for resistance

Preparation of inoculum of *P. infestans* (races 0, 1, 3, 4, 1.4, 4.10 and the complex race 1.2.3.4.5.6.7.10.11) and the inoculation procedure were carried out as described previously (El-Kharbotly et al., 1994). The fungal races were kindly supplied by Dr. L.J. Turkensteen (Research Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands) and Dr. F. Govers (Department of Phytopathology, Wageningen Agricultural University , The Netherlands).

The disease assessment was made five days after inoculation. Plants were scored as: (1) resistant (R) when symptoms were absent or a hypersensitive reaction was visible; and (2) susceptible (S) when sporulating lesions developed. The resistance test was repeated twice. Parents, as well as defined genetic material resistant to specific races of *P. infestans* (C. Mastenbroek unpublished: tetraploid tester clones containing *R0, R1, R3, R4,* and *R10* loci), were used as controls in both tests.

Ploidy level and fertility determination

Ploidy level of the potential dihaploids was determined by counting the number of chloroplasts in the stomatal guard cells (Frandsen, 1967)

Male fertility of the plants was estimated after staining pollen with lactophenol acid fuchsin.

Molecular analysis

Parental clones (87-1024-2 , J91-6167-2) and their F_1 (family J92-6400-A) were used for RFLP analysis. DNA extraction (Dellaporta et al., 1983), restriction digests, electrophoresis, blotting and hybridization procedures were done as recommended by suppliers. The RFLP probe GP21, linked to locus *R1* (Leonards-Schippers et al., 1992), was used for hybridization of the Southern blots. The RFLP probe was kindly supplied by Dr. C. Gebhardt (Max-Planck-Institut Für Züchtungsforschung, Köln, Germany).

Results

Alteration of specificity of resistance in sexual offspring

R4

The tetraploid clone J90-6028 was tested with the set of races of *P. infestans* and showed, as expected, specificity for *R4*. This was demonstrated by resistance to the races 0, 1, 3 and susceptibility for the races 4, 1.4 and 4.10. The production of dihaploids from this clone using *S. phureja* was not successful due to the short flowering period. This problem was overcome first by crossing J90-6028 with the susceptible tetraploid clone J90-6001-4. The well flowering F_1 progeny (coded J91-6148) was screened with *P. infestans* race 0 for segregation and showed an excess of susceptible phenotypes 16:32 (resistant (R) versus susceptible (S); $X^2 1:1 = 5.33$, $P < 0.05$). The resistant F_1 plants were additionally tested with race 4. They displayed susceptibility, confirming that the resistance is specific to the genetic factor of *R4*. Three resistant clones J91-6148-4, -14 and -24, were used as alternative 4x parents for dihaploid induction with *S. phureja*. These three clones gave the same reaction pattern, with the tester set of *P. infestans*, as the earlier tested parental clone J90-6028. In total 62 dihaploids were isolated and tested from which 42 showed resistance to race 0 (Table 1A). Surprisingly, the dihaploids from all three tetraploid parents showed an excess of resistant individuals. In two of the three progenies with dihaploids a 1 : 1 segregation was found. In the larger progeny J92-6427 an excess of resistant plants was significant. All 42 resistant dihaploids, originating from the tetraploids J91-6148-4, -14 or -24, were tested as female in a crossing programme with susceptible diploid pollinators. Only the dihaploid J92-6427-4 gave seed set with both of the diploid males 87.1024-2 (60 seeds) and IVP 48 (100 seeds) resulting in the two families BE93-4030 and BE93-4031, respectively. This dihaploid was also tested with the races 4, 1.4 and 4.10. Unexpectedly, it showed resistance instead of susceptibility suggesting the expression of another genetic factor R_n. This is our second clear example showing a change in specificity of resistance after extracting parthenogenetic dihaploids using a *S. phureja* clone which was susceptible to all tested races of *P. infestans*. The first example of the phenomenon of an altered reaction pattern to races of *P. infestans* was described earlier in a dihaploid from *cv.* Hertha (El-Kharbotly et al., 1994).

The germination rate of the seeds from this genotype was low giving rise to only 13 seedlings in the two families. Of these, six developed to maturity and could be tested with *P. infestans* enabling the selection of three resistant plants. All three

Table 1 Segregation of tested dihaploids, and F_1 populations after inoculation with *P. infestans* race 0. The F_1 populations were obtained after crossing resistant dihaploids or diploids with susceptible diploids.

Resistant parent Code	Genotype[a]	F_1 population (code)	Test with race 0 Number of plants R	S	X^2 1:1
A. *R4*					
Dihaploid induction :					
J91-6148- 4[b]	*R4*	J92-6027[c]	21	8	5.83*
-14	*R4*	J92-6029	12	6	2.00
-24	*R4*	J92-6031	9	6	0.6
Total			42	22	
Dihaploids					
J92-6427-4	R_n	BE93-4030	1	1	nt
J92-6427-4	R_n	BE93-4031	2	2	nt
Diploids					
BE93-4031-1	R_n	BE94-4100	51	83	7.64**
BE4100-106	R_n	BE95-4211	34	21	3.07
B. *R10*					
Dihaploid induction :					
Primeri[b]	*R10*	J92-6566[c]	10	6	1.00
Dihaploids					
J92-6566-3	*R10*	BE94-4103	1	57	54.07**
J92-6566-17	*R10*	BE94-4102	13	95	62.26**
Diploids					
BE94-4102-121	R_i	BE95-4209	10	47	24.02**
BE94-4102-150	R_i	BE95-4210	2	42	36.36**
BE94-4103- 52	R_i	BE95-4208[d]	21	38	4.90**

nt, not tested
a, proposed genotype based on resistance pattern using the tester set of *P. infestans* and each of the resistant parents
b,c, Tetraploid clones/cultivars and dihaploids respectively
d, segregating for R_i and *R10*
*, **, significantly different from the expected ratio at $P<0.05$ and $P<0.01$, respectively.

plants with resistance to *P. infestans* race 0 showed also resistance to race 4 (Table 1A). Only the individual resistant plant, BE93-4031-1, gave seed set after pollination with the diploid susceptible clone 87-1024-2. A high frequency of susceptible phenotypes was observed in the F1 progeny BE94-4100 of this clone (Table 1A) using *P. infestans* race 0. Twenty six resistant plants of this F_1 progeny were additionally tested with the tester set for the presence and expression of only *R4*. One of them expressed only the *R4* gene and the other 25 showed the earlier described altered specificity which is probably based on one unknown *R*-gene. Unfortunately, the *R4* specific plant BE94-4100-131 showed slow growth and did not flower. Therefore, crossing was only successful with seedling BE94-4100-106, one of the other 25 resistant plants. Its progeny BE95-4211 showed after inoculation with *P. infestans* race 0 a segregation of 34 resistant and 21 susceptible plants fitting a 1 : 1 segregation ($X^2$1:1 =3.07, P> 0.05; Table 1A). Race specificity tests did not show the appearance of *R4* specific segregants. The plants with an altered resistance pattern gave susceptibility to the complex race of *P. infestans*. More tests with different race combinations of the fungus are needed to identify which specific *R*-gene(s) were additionally available in the originally resistant plant J90-6028 or in one of the susceptible crossing parents.

R10

Cultivar Primeri, containing *R10*, gave after testing with the *P. infestans* races 0, 1.4 and 4.10 the expected reaction pattern. Induction of dihaploids was successful. Sixteen dihaploids were isolated and tested for resistance and no difference with a 1:1 segregation was found (Table 1B). Testing for specificity of resistance with races 1.4 and 4.10 indicated only the expression of *R10*. After crossing with the susceptible diploid clones 87-1024-2 and J89-5040-2 sexual offspring was obtained from the resistant dihaploids J92-6566-3 and J92-6566-17. In family BE94-4103 only one out of 58 seedlings and in BE94-4102, 13 out of 108 seedlings were resistant to race 0. These plants were also resistant to both races 1.4 and 4.10 indicating a change in specificity of resistance. This phenomenon was still present in the offspring after crossing of the resistant genotypes BE94-4102-121, -150 and BE94-4103-52 with the susceptible diploid clone SUH3711. In all three families a high degree of distorted segregation with excess of susceptible plants was found using *P. infestans* race 0. The resistant plants were additionally tested with *P. infestans* races 1.4 and 4.10. In family BE95-4208 a clear segregation for race specificity was found, seven out of 21 resistant plants appeared to be susceptible to race 4.10 indicating the expression of the

resistance gene *R10*.

Expression of the R1- specific resistance factor in offspring of crosses between susceptible plants

The change in specificity of resistance in the above described material, containing the genetic factors *R4* or *R10*, can be explained by the existence and segregation of a second genetic factor in the genome influencing expression of "unidentified" *R*-genes. The application of markers closely linked to *R*-genes in combination with expression of these *R*-genes could be used to answer the question whether this second genetic factor can be detected influencing expression of "known" *R*-genes or not. The resistance genes *R1*, *R3*, *R6*, and *R7* are useful for answering this type of questions, because they have been localised by RFLP markers in known material (El-Kharbotly et al., 1994 and chapter 2).

The above mentioned possibilities were investigated in diploid potato material bearing the resistance gene *R1*. The RFLP marker *GP21* is closely linked to this *R1* gene varying in distance between 2.5 and 4.5 cM (Leonard-Schippers et al., 1992; El-Kharbotly et al., 1994). The progeny of the family J92.6400-A, segregating for *R1* and the parental clones J91-6167-2 and 87-1024-2, were used for these studies. Seventy plants of this family were tested for the potential recombinations between the allele of probe *GP21* and the *R1*-gene. Ten susceptible "recombinants" were found containing the (*R1*-linked) *GP21* allele. Four of them gave sufficient seed set when crossed with the susceptible parent SUH3711 . The seedlings of these families were tested for the expression of resistance to *P. infestans* race 0 (Table 2). As expected, in the F_1 progeny of one combination (J92-6400-A-50) only susceptibility was found. However, in the F_1's from the other three combinations (J92-6400-A-91, -92 and -93)resistant plants appeared. The frequency of resistant plants within the populations varied between 18 to 63%. Additional tests for resistance with the races 1.4 and 4.10 indicated that in this material the resistance factor *R1* was active. These results clearly indicate that the above predicted two possibilities of susceptibility based on presence or absence of the second genetic factor or on recombination could be found. This means that because of this additional possibility resistant offspring plants can be obtained in crosses between susceptible parents when *R*-genes are present in combination with genetic factors suppressing expression of resistance.

Table 2 Segregation for resistance to race 0 of *P. infestans* in several F_1 populations of crosses between susceptible diploid parents. The female parents were containing the RFLP allele GP21 which is linked to the resistance gene *R1*.

Diploid parent	F_1 population (Code)	Test with race 0 Number of plants		
		R	S	$X^2$1:1
r1r1[a]				
J92-6400-A-50	BE95-4213	0	30	30.00**
-91	-4214	8	15	1.07
-92	-4215	5	23	11.57**
-93	-4216	5	3	nt
Total		18	71	

nt, not tested
a, proposed genotype based on susceptibility to race 0 of *P. infestans*
**, significant at $P<0.01$

Discussion

The vertical resistance against specific races of *Phytophthora infestans* is normally described to be controlled by single dominant resistance genes (*R*-genes; Mastenbroek, 1953; Malcolmson and Black, 1966). Here we demonstrate the presence of additional genetic factors involved in the expression of these *R*-genes. The increase of partial resistance level or the extension to more races of *P. infestans* after dihaploid induction using the prickle pollination was reported earlier but not investigated (De, Maine, 1978; El-Kharbotly et al., 1994). Also other examples of increased pathogen resistance in dihaploids, than originally found in their parents, have been described for the potato cyst nematode (*Globodera pallida*) (De, Maine, 1978). In all these cases a few dihaploids were recovered that showed this phenomenon. This alteration may be caused by the removal of a genetic factor(s) by meiotic recombination which was suppressing the expression of an *R*-gene(s) already present in the original tetraploid. Wilkinson et al. (1995) demonstrated some evidence for somatic translocation during potato dihaploid induction using *Solanum phureja* as the pollinator. Therefore not only removal of a suppressor can explain this phenomenon but also the introduction of

genetic material from *S. phureja* enabling expression of the resistance factor. Both possibilities can be involved to explain the altered resistance specificity which was found in the dihaploid J92-6427-4 (Table 1A; *R4*).

In tomato, two loci, *Rcr*-1 (required for *Cladosporium* resistance) and *Rcr*-2 (for *Cladosporium* resistance), have been identified which are required for optimized race-specific resistance of the *Cf*-9 gene to the fungal pathogen *Cladosporium fulvum*. These genes are different from each other and from *Cf*-9 (Hammond-Kosack et al., 1994). Also, Freialdenhoven et al. (1994) reported that *Nar*-1 (necessary for Mla_{12} resistance gene) and *Nar*-2 are two loci required for expression of Mla_{12}-specified race specific resistance to powdery mildew in barley. There was, however, an important difference in effect between mutant alleles of *Rcr*-1 and *Rcr*-2 in tomato and mutant alleles of *Nar*-1 and *Nar*-2 in barley. Defective alleles of *Rcr*-1 and *Rcr*-2 weakened the resistance response of *Cf*-9 without permitting sporulation. Mutant alleles of *Nar*-1 and *Nar*-2 allowed sporulation and, therefore, completion of the *Erysiphe graminis* f sp *hordei* life cycle.

In potato, the segregation analysis and the probabilities associated with X^2 values strongly supported the hypothesis that resistance to infection by PVY or PVX was controlled by complementary action of at least two dominant genes (Vallejo et al., 1995). If two genes are also involved in the expression of race specific resistance to *Phytophthora infestans* in potato, it is expected that in certain crosses of susceptible segregants to susceptible tester plants segregation of resistant plants will occur as shown in Table 2. These ones are indications for the involvement of a second factor.

The existence of above described factors in barley and tomato has been investigated by mutation induction using ethyl methanesulphonate (EMS) in resistant genotypes (Torp and Jorgensen, 1986; Hammond-Kosack et al., 1994). In our case, the use of RFLP markers appeared to be a powerful tool for molecular marker based selection of susceptible plants which could be the result of a second genetic factor suppressing the expression of *R1*. In the offspring of test crosses with susceptible plants lacking expression of *R1*, in three of the four cases resistance was found. This observation indicates that the second genetic factor is expected to be a suppressor gene. The second conclusion is that in mapping of *R*-genes misclassification can be made causing over estimation of the frequency of recombinants. The biological assay of testing for presence or absence of the *R1* gene is inconclusive when the second factor is segregating in the same population. Test crosses with plants recessive for both genes could give more reliable information about the genotype of susceptible plants with respect of the *R1* allele. It is clear from our results that one of the factors causing distorted segregation of race specific *R*-genes is the second genetic factor

which in the case of *R1* could be a dominant suppressor. Crossing of indirectly selected susceptible plants with recessive tester clones, as applied by van Eck et al., (1994) in studies on flower and tuber skin colour, could help in localisation of the second genetic factor.

The observations with the *R4* and *R10*-containing materials are less conclusive. Enhancers or suppressors can be involved in the expression in the offspring of R_n and R_i loci which probably were already present in the primary resistant clones PI 203900 and cv Primeri. In future, genotypes with the suppressor factor are important for investigating the expression of the cloned *R1* gene which can be introduced by transformation.

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Chapter 7

General discussion

Potato (*S. tuberosum*) is an autotetraploid (2n=4x=48) crop plant with a high level of heterozygosity. The high ploidy level makes all kind of genetic research such as localisation of (molecular) markers on the genome or transposon tagging by insertion mutagenesis difficult. These problems can partly be solved by investigation of the cultivated potato at the diploid level. However, at the diploid level a gametophytic incompatibility system is active and restricts the possibility of selfing. Parthenogenesis, using specific clones from the cultivated primitive species *S. phureja* (Hermsen and Verdenius, 1973) as pollinator, enables the reduction of the tetraploid to the diploid level (dihaploid induction; 2n=2x=24). The success rate of this method of dihaploid induction is highly dependent on the genotype of the tetraploid *S. tuberosum* female and the *S. phureja* male parents (Hutten, 1994). Short flowering period and low fertility also had a negative influence on the dihaploid induction (Chapters 2, 3 and 6)

Development of diploid populations with predictable segregation for *R*-genes

Diploid potato populations were needed for two main purposes:

1. genetic localisation of *R*-genes giving rise to race specific resistance to *Phytophthora infestans* by RFLP-mapping (chapters 2 and 3)
2. improvement of regeneration and transformation efficiencies in diploid potato for the selection of resistant and fertile potato clones suitable for transposon tagging research (Chapter 4).

As indicated in this thesis, different problems were associated with the realisation of both purposes.

Dihaploid induction was, in contrast to many potato cultivars, difficult in most of the tetraploid differential clones bearing specific *R*-genes (chapters 2, 3 and 6). The differential clones generally had a low fertility and a short flowering period probably because of the presence of a relatively large amount of genetic information of the donor species *S. demissum*. After introduction of *R*-genes by interspecific hybridisation probably only a few backcrosses with *S. tubersosum* were made for the selection of differential clones expressing one *R*-gene. The problem of flowering and seed set could be overcome by additional crossing with tetraploid susceptible pollinators which were well flowering and highly fertile (chapters 3 and 6). However, these crossings at the tetraploid level were possible with the differential clones MaR4 and MaR6 but

more difficult in combinations with the differential clones MaR2, MaR5, MaR8 (data not shown). The tester clone MaR9 appeared in our hands to express more resistance factors than only *R9* (data not shown). This clone needs further testing in sexual offspring to separate *R9* from the other resistance factors before *R9* can be localised.

The problems associated with development of useful dihaploid plants were malformation, low vigour and low fertility, which may be explained by expression in dihaploids of unfavourable recessive alleles present in the original tetraploid parent and inbreeding. Within the populations of dihaploids biased segregation for the *R*-genes was frequently found towards susceptibility from the expected 1:1 or 3:1 ratios. This observation is different from the segregation behaviour in dihaploids of the resistance gene *Ro-1* against *Globodera rostochiensis* Woll. (Hutten et al., 1995) which showed the expected ratio. Skewed segregation of R-genes was also frequently observed, after crossing of dihaploids with diploids, in many diploid families while a smaller proportion gave clear cut segregation ratios. The same kind of biased segregations were earlier reported in diploid populations for resistances to virus X (*Rx2*) (Ritter et al., 1991) as well as for the blue anthocyanin locus *P* (van Eck et al., 1993). In case of locus *P*, mapped on chromosome 11, skewedness was also observed for the linked RFLP markers. In our material, *R3* was mapped on the same chromosome 11 showing also a biased segregation (chapters 2). However, in the same plant material inheritance of *R1* on chromosome 5 and *amf* on chromosome 8 behaved normal. Further it was noticed that clear cut segregations for resistance to races of P. *infestans* could be observed in sexual progeny from biased individuals and vice versa (data not shown). These changes of genetic behaviour in subsequent generations could be an indication for the presence of other genetic factors influencing the expression and inheritance of this type of resistance.

Sometimes altered race specificities were observed in plant material at the dihaploid or diploid level which were not found in the tetraploid or diploid parental plants (chapters 2 and 6). Altered reaction patterns than found in the parents were earlier observed and described in dihaploids by De, Maine, (1978) for resistance to nematodes and *P. infestans*. This observation indicated two phenomena 1) presence and expression of hidden (unidentified) resistance genes and 2) expression of *R*-genes can be dependent on other genetic factors. Our observations focused on expression or absence of expression of *R1* suggest the same phenomena but now related to a known *R*-gene. This means that either a suppressor or an enhancer gene is involved in the expression of the race specific resistance factor. The existence of additional genetic factors for (better) expression of resistance genes is well known and studied in more detail in wheat (Kerber, 1991), barley (Freialdenhoven et al., 1994) and tomato

(Hammond-Kosack et al., 1994). Next step is localisation of this genetic factor in potato and investigation of the nature of it. A required population will be developed in such a way that transposon tagging of this genetic factor can be applied. Mapping of *R*-genes is less straight forward when the genotype of the plant is estimated by the phenotypic reaction to races of *P. infestans* and not by test crosses.

Homozygous plants

After intercrossing of heterozygotes, containing *R1* or *R3* respectively, eleven and eight segregating populations were developed to select for homozygous genotypes *(R1R1* or *R3R3)*. Despite the application of many test crosses, only three homozygous *R1R1* plants were selected from two populations. It is not known whether the presence of the above described "other" genetic factor involved in the expression of *R*-genes was the negative key factor or not. Homozygous genotypes are needed in our transposon tagging strategy (chapter 1). However, an additional step has to be made to check whether the genotypes involved in transposon tagging does not contain other genetic factors delivering false negatives. This is specifically a problem when this genetic factor is closely linked to the *R*-gene of interest.

Localisation of *R*-factors

It was possible to localise *R*-genes either from clearly segregating populations containing *R1* and *R3* or from populations with skewed segregation of *R6* and *R7.* Surprisingly, three *R*-factors *R3*, *R6* and *R7* were mapped at the distal part of chromosome 11 (chapters 2 and 3) linked to the same RFLP markers. It is not clear from our data or from earlier pedigree whether they are alleles or not. Further research may reveal whether *R3*, *R6* and *R7* are either allelic, part of a complex locus or different loci. Mapping of *R1* on chromosome 5 clearly indicated that it is not allelic to any of the other three *R*-genes.

Regeneration and transformation competence

As has been reported earlier, a factor for transformation competence could be transferred from the wild species *Lycopersicon peruvianum* to the cultivated tomato *L. esculentum* (Koornneef, 1986). The same appears to be true for potato. It was possible to improve transformation efficiency and regeneration ability, as well as the speed of regeneration and regeneration on both cut surfaces of stem explants. The

transformation competence factor was localised on chromosome 5 linked to *R1* and to the RFLP marker *GP21*. On the other hand, not all varieties bearing *R1* were easy to transform, (unpublished data).
For our transposon tagging work the well transforming *R1r1* clone J92-6400-A16 has been selected. This diploid clone was additionally used as a donor for transformation competence in research programs on successful modification of gametophytic incompatibility by (anti)sense genes coding for *S*-alleles (Eijlander personal comm.).

Insertion mutagenesis of *R*-genes

T-DNA mediated gene tagging in Arabidopsis (Lönneborg and Jansson, 1993) has the advantage that it induced stable mutants in one step. This is not the case in using autonomous transposable elements like *Ac*. A disadvantage of T-DNA mediated gene tagging is the very large number of transformants which is required to create a reasonable chance to tag a particular gene in a relatively large genome like potato. The use of a non-autonomous transposable element like *Ds* (which can be mobilized by introduction of a transposase gene) in T-DNA is reducing the number of transformants needed for tagging of genes and enabling, after removal of the transposase gene by crossing, selection of stable mutations. The population of 60 diploid transformants with the localised *Ds*-containing T-DNA inserts, which are randomly distributed over all 12 chromosomes, is an important first step to reach this goal. These transformants are a notable outcome of this thesis. These transgenic plants are the basis for tagging research in potato not only of *R*-genes but in general for other genes of interest. Research has been started to tag genes which are involved in metabolic pathways.

The *Ds* element can be activated by introducing the *Ac* transposase gene by transformation or stabilised by removing this transposase source through out-crossing and segregation. It has been reported that the *Ds* element has the tendency to transpose after excision to neighbouring locations of the same chromosome not only in maize but also in tomato (Knapp et al., 1994; Thomas et al., 1994). In tomato other examples are also known with predominant transposition to other chromosomes (Healy et al., 1993; Rommens et al., 1993). Therefore, transposition behaviour of selected potato genotypes has to be known before tagging experiments can be started. In our experiments as much as 377 diploid *Ds* containing transformants were produced from which only 18% could be localised. In two transformants T-DNA inserts were linked in repulsion to *R1*. After a crossing step recombinants could be selected with a true linkage between the *R1* allele and the *Ds*- containing T-DNA. They are being used in another project for the second step which is retransformation with the *Ac* transposase

gene in order to mobilise *Ds*, which will be followed by tagging and cloning of the *R1*-gene. In other *Ds* containing plants we have already observed that *Ds* can be mobilised by the transposase gene used here.

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Summary

The late blight disease caused by the fungus *Phytophthora infestans* (Mont) de Bary, is one of the most serious diseases of potato. It causes a considerable reduction of the tuber yield. The infected tubers not only carry the disease from one season to another but they also can infect the neighbouring tubers during storage resulting in high post-harvest losses.

One of the methods to control this disease is the repeated use of fungicides. This method is not only costly but is also a great source for the environmental pollution in the Netherlands. A weather forecasting system, to predict the suitable condition for the fungal infection of the crop, so that the chemical control could be applied efficiently, is a way to reduce both the costs and the pollution.

Using resistant varieties is another method for protection of the crop against this disease. The wild potato germplasm is used as a source for resistance in many breeding programs. Resistance against *P. infestans* is classified as either horizontal, which is a polygenic trait, or vertical. The vertical resistance is controlled by single dominant genes, known as *R*-genes. The presence of *R*-genes protects the plant against the pathogen through a hypersensitivity reaction. In the past, eleven *R*-genes, conferring resistance to specific races of *P. infestans*, were introduced to the cultivated potato (*Solanum tuberosum*) from the hexaploid wild species *S. demissum*.

On the other hand, the fungus showed the ability to develop new adapted races either during its vegetative or its sexual reproduction. It developed resistance to the fungicides and it was able to break genetic resistance factors in the plant. Both aspects make it difficult to control this disease.

Molecular cloning of *R*-genes is of both practical and theoretical interest to control this disease. The resistance mechanism can be unravelled probably enabling the development of strategies leading to more durable resistance. Homology between different *R*-genes can result in their use in screening methods when *R*-gene free breeding material is needed and in the isolation and combined application of new *R*-genes present in other unexploited accessions of *S. demissum*. Isolation of *R*-genes via protein characterization and cDNA cloning is not possible because this gene product is not known. An alternative approach is thought to be through insertion mutagenesis using transposable elements (transposon tagging). In the last decade it became clear that in plant species like maize and *Antirrhinum*, genes could be identified and isolated by insertion mutagenesis of (non)-autonomous transposable elements. The *Ac-Ds* system of maize, consisting of the non-autonomous *Dissociation (Ds)*-element and the

autonomous *Activator (Ac)*-element (also activating the *Ds*-element), could after molecular isolation be successfully transfered to other plant species. In tomato and potato the introduced *Ac*-element is very active and the *Ds*-element could be activated by the *Ac* transposase. It was known from maize that *Ac/Ds* elements mainly transposed to closely linked positions. For the application of this approach in potato, both the knowledge of the position of the desired *R*-gene on the potato genome and the presence of a transposon-carrying T-DNA near the *R*-gene are essential. Also the tetraploid nature of the potato necessitates the use of diploid potato genotypes for genetic analysis as well as transposon tagging. In the last decade, the Restriction Fragment Length Polymorphism (RFLP) technique enabled the construction of genetic maps of different crops including potato. Detection of linkage between an *R*-gene and particular RFLP markers determines its position on a particular potato chromosome. The map position of the *R*-genes, linkage relationships or possible allelism were at the start of the project not known, with exception of the *R1* gene which was located on chromosome 5. The research which is reported in this thesis involved genetic studies on and localisation of different *R*-genes as well as development of resistant plant material suitable for transformation and targeted transposon tagging of these genes at the diploid level. The first step was to reduce the ploidy level starting with tetraploid varieties and differentials expressing particular *R*-genes.

From ten tetraploid potato cultivars bearing one or more *R*-genes (*R1, R2, R3, R10*), 157 parthenogenetic dihaploids were isolated for the purpose of localization of these genes (Chapter 2). Most of these dihaploids showed malformation of seedlings and low vigour. They were screened for resistance to *P. infestans* race 0 to determine the presence or absence of expression of *R*-genes. In the dihaploid progeny of cultivars bearing *R*-genes the frequency of resistant plants was much lower than expected. After grafting onto tomato rootstocks, only eight out of 60 resistant dihaploids set seeds. Detailed screening of these eight clones for race specificity indicated the presence of *R1*, *R3*, *R1R3*, or *R1R3R10*. One clone (Esc.42: originating from *cv* Escort) contained *R1R3* and another clone (Her.64: originating from *cv* Hertha), surprisingly, showed besides *R1R3R10* an additional *R*-gene specificity which was not expressed in the original tetraploid parent. Nineteen F_1 progenies of the eight resistant dihaploids were screened for the expression of resistance, using race 0 of *P. infestans*. Defined fungus isolates were additionally needed for the segregation analysis of particular *R*-genes. Distorted segregation in favour of susceptibility was found for the *R3* gene in 15 out of 17 F_1 populations, whereas the *R1* gene segregated as expected in five F_1 populations. Independent segregation of *R1* and *R3* was demonstrated in two F_1

populations of crosses between the dihaploid Ecs.42 and susceptible pollinators. One of these two F_1 populations, J91-6165, showed a deficit of genotypes expressing *R3*. On the other hand, *R1* segregated as expected. The second F_1 population J91-6164 which showed a clear cut segregation for both *R1* and *R3*, was used successfully for RFLP analysis. Linkage analysis between *R1*, *R3* and RFLP markers of known positions on the potato genome confirmed the position of the earlier mapped *R1* gene on chromosome 5 and mapped *R3* to a distal position on chromosome 11.

In further localization research on other *R*-genes, except *R1*, *R3* and *R10*, the differential clones MaR2, MaR4, MaR5, MaR6, MaR7, MaR8, MaR9 and MaR11 were used. the differential clones, each expressing a specific *R*-gene, have been developed in the past for race identification of isolates of *P. infestans*.

Only the tetraploid differential clones MaR6 and MaR7, bearing *R6* and *R7* respectively, could successfully be used as parental clones (Chapter 3). Three dihaploids were isolated from MaR6. They did not set seeds after crossing with diploid susceptible pollinators. To overcome this problem of sufficient dihaploid induction, the clone MaR6 was crossed with a susceptible tetraploid pollinator. The F_1 population showed a 5:1 segregation ratio (resistant versus susceptible) after inoculation with *P. infestans* race 0. This indicated that *R6* in MaR6 was present in duplex form. Five resistant F_1 progeny genotypes were selected and used as parents for dihaploid induction. In total 42 dihaploids were isolated from which 29 were resistant containing *R6*. The induction of resistant dihaploids from MaR7 was possible. Six dihaploids were isolated from which two appeared to be expressing *R7*. The same phenomenon of low male and female fertility for dihaploids, which has been mentioned earlier, was found in the dihaploids containing either *R6* or *R7*. In crosses with susceptible diploid tester clones six and one dihaploid(s) containing *R6* and *R7* respectively, set seeds. In general, as found for *R3*, an excess of susceptible genotypes was found in these F_1 progenies. On the other hand no altered *R*-specificity was found. RFLP analysis with probes linked to either *R1* or *R3* revealed a clear linkage of both *R6* and *R7* to the same probes that linked to *R3* on chromosome 11. The recombination behaviour and frequency, between the molecular markers and these three *R*-genes, were not the same. The RFLP recombinants in the F_1 populations containing *R3* or *R7* were few but within the class of susceptible genotypes, whereas those in the F_1 population containing *R6* occurred more regularly within the class of resistant genotypes. From our data, it is not clear whether *R3*, *R6* and *R7* are allelic or belong to a complex locus.

Transformation is the first step in introducing the non-autonomous maize transposable element *Ds* to potato for the purpose of targeted transposon tagging. Appropriate diploid potato genotypes have to be developed for tagging strategies. Linkage between *Ds*-containing T-DNA-inserts and *R*-genes can only be detected when a great number of transformants is available for selection. This means that well-transforming diploid genotypes, preferably bearing the *R*-gene of interest, should be selected (chapter 4). The competences for regeneration and transformation were investigated in 11 resistant diploids (*R1r1*). Regeneration percentages in these genotypes varied between 55% and 95%. Screening for transformation showed that only one clone (J91-6167-2), derived from *cv* Hertha, gave high transformation efficiency (TE; 24%). This clone, however, was male sterile and the berry and seed set was low. To overcome this fertility problem it was crossed with another susceptible well-transforming pollinator (87-1024-2). The F_1 progeny was screened for regeneration and transformation ability as well as for resistance to *P. infestans* race 0. For transformation *Agrobacterium tumefaciens* strain AM8706, containing the NPTII gene for kanamycin resistance and the reporter gene *β*-glucuronidase (GUS), was used. This F_1 population, of 26 plants, segregated for regeneration ability, speed of regeneration and the regeneration from both cut surfaces on the stem explant. A few genotypes showed higher values for these characters than the parents. Segregation was also found for transformation efficiency. Traits like fast regeneration and regeneration from both cut surfaces on stem explants after transformation, under selective growth conditions with kanamycin, were found in four and eight genotypes respectively, while they were not present in the parental clones. Based on all these results a well-transforming and fertile genotype, J92-6400-A16 was selected. This clone yielded predominantly diploid transformants and was heterozygous for the *R1* gene, conferring resistance to *P. infestans*. Linkage was found between competence for transformation and both the *R1* locus and a molecular marker on chromosome 5. It was also observed that the female fertility, of the transformants, was less affected by transformation than the male fertility.

For the purpose of tagging the *R1* gene by using the maize transposable element *Dissociation* (*Ds*), the clone J92-6400-A16 was transformed with the pHPT::*Ds*-Kan construct (Chapter 5). The T-DNA of the construct consisted of the kanamycin resistance gene (*NPTII*)-containing the *Ds*-element which was placed between the promotor and the structural gene coding for hygromycin resistance (*HPT*). Selection of transformants was based on kanamycin resistance as the selectable marker (Kan). Hygromycin resistance (HPT) was used to monitor excision of the *Ds* element

which after a second transformation was induced by the *Ac* transposase gene. From 400 stem explants, inoculated with *Agrobacterium tumefaciens*, 377 transformants were obtained from which 65 appeared to be tetraploid. Thus, 312 diploid transformants were used for molecular analysis. In 24% of them a single copy of T-DNA was integrated while 2-8 copies were present in the rest. Genomic DNA sequences flanking the T-DNAs from 187 transformants were isolated as probe using Inverse Polymerase Chain Reaction (IPCR) or Plasmid Rescue (PR) techniques. From 216 isolated probes, 96 were polymorphic and 60 were mapped. Detailed analysis of T-DNA integration in the genome of diploid potato could be made. Based on the physical chromosome length and/or euchromatin content the T-DNA insertions were found to be distributed randomly on all 12 chromosomes. This random distribution was also found when the chromosome length, based on the RFLP map of the mapping population, was used in the calculation. The T-DNA insertions of two transformants, BET92-Ds-A16-259 and BET92-Ds-A16-416, were linked in repulsion to the *R1* gene. Genetic analysis of the F_1 progeny of BET92-Ds-A16-416, after crossing with a susceptible parent, confirmed the linkage between the inserted T-DNA and the *R1* gene. Four recombinants (*Ds*-carrying T-DNA linked in coupling phase with *R1*) were discovered. They are the basic plants for the next step in tagging the *R1* gene. Mobilization of the *Ds* element after introducing the *Ac* transposase gene, through transformation, was successful in four tested transformants containing a single copy of T-DNA.

In the course of developing diploid populations for RFLP analysis an alteration of ***R*** specificity and excess of susceptible genotypes were observed (Chapter 2). The phenomenon of changing race specificity was investigated in more detail using parental clones containing *R4* or *R10*. A clear evidence for involvement of a second genetic factor in the expression of (unidentified) *R*-genes was found (Chapter 6).

The production of dihaploids from the tetraploid clone J90-6028, containing *R4*, was not successful due to the short flowering period. As has been mentioned earlier for clone MaR6 (Chapter 3), this problem could be overcome by crossing with a tetraploid susceptible pollinator. The F_1 progeny showed an excess of susceptible genotypes but no alteration of *R* specificity in the resistant genotypes. From three tetraploid progeny clones 64 parthenogenetic dihaploids were obtained after crossing with *S. phureja*. Surprisingly, an excess of resistant dihaploids was observed after inoculation with *P. infestans* race 0. Regarding the low fertility of the dihaploids, only one set seed in crosses with two susceptible male parents. Determination of the phenotype of this dihaploid for resistance showed an alteration of specificity i.e.

resistance to race 0 and 4 instead of only to race 0. Three out of six plants from these two F_1 populations were again resistant to the same races of *P. infestans* but only one of them (BE93-4031-1) set seed with a susceptible diploid pollinator. The F_1 progeny of BE93-4031-1 with many seedlings showed an excess of susceptible phenotypes. Determination of race specificity among 26 resistant plants showed that only one plant was *R4* specific and that the other 25 still contained the altered specificity. Unfortunately, crossing was not possible with this *R4*-specific genotype. Crossing of another resistant plant from the same F_1 population with altered specificity showed a 1:1 segregation ratio with *P. infestans* race 0, and no *R4*-specific plants.

Sixteen parthenogenetic dihaploids from *cv* Primeri, containing *R10*, showed the expected segregation ratio 1:1. Also testing for race specificity indicated the expression of only *R10* in the resistant dihaploids. The segregating F_1 populations from two of these resistant dihaploids showed for all resistant plants alteration in race specificity (resistance to race 4.10) indicating the presence of an unidentified *R* factor(s). A clear segregation for race specificity was found in one of the families. Seven out of 21 resistant plants appeared to be susceptible to race 4.10 indicating R10 specificity. This phenomenon of changing specificity can be explained by the existence and segregation of a second genetic factor influencing expression of the unidentified *R*-gene.

The existence of such a second genetic factor influencing the expression of a known *R*-gene was studied in relation to *R1*. Ten susceptible "recombinants" still containing the RFLP allele of probe *GP21* which is known to be linked to the *R1* allele were used in crosses with a susceptible pollinator. Four of these selected plants set seed. Genotypes resistant to *P. infestans* race 0 were recovered in three out of the four F_1 progenies. Testing for race specificity indicated the expression of the *R1* allele in these plants. This is a clear indication that the *R1* allele was not expressed in the parental clones either due to the presence of a suppressor or as a result of complementary gene action. Further research is needed to localise and to determine the nature of this unidentified factor.

From the data described, it is clear that *R*-genes can be localised and tagged in diploid plant material. The *R3, R6* and *R7* alleles can be part of a complex locus, allelic or belonging to different loci. In potato, the detection of a second genetic factor involved in the expression of *R*-genes is complicating genetic studies on resistance to *P. infestans* and practical breeding. The selection of a well transforming *R1r1* diploid enabled the development of hundreds of transformants containing the *Ds*-containing T-DNA. The 60 transformants with localised *Ds*-containing T-DNA inserts distributed

all over the potato genome can be used not only for tagging of *R*-genes but also for the isolation of genes coding for other traits. The competence for transformation could successfully be transferred to other diploid potato genotypes.

Samenvatting

De aardappelziekte die door de schimmel *Phytophthora infestans* (Mont.) de Bary veroorzaakt wordt, is één van de ernstigste ziekten bij dit gewas. Het veroorzaakt een aanzienlijke reductie van de knolopbrengst. De geïnfecteerde knollen dragen de ziekte niet alleen over van het ene naar het andere seizoen maar infecteren bij bewaring ook naburige knollen waardoor grote opslagverliezen ontstaan.

Eén van de methoden om deze ziekte in de hand te houden is het herhaald gebruik van gewasbeschermingsmiddelen. Deze methode is niet alleen duur maar ook belastend voor het milieu in Nederland. Een waarschuwingssysteem waarmee gunstige weersomstandigheden voor schimmelinfectie op dit gewas voorspeld kan worden, is een manier om zowel de kosten van de bestrijding als de milieubelasting te reduceren.

Het gebruik van resistente rassen is een andere methode om dit gewas tegen deze ziekte te beschermen. Wilde aardappelsoorten worden als bron van resistentie in veel veredelingsprogramma's gebruikt. Resistentie tegen *P. infestans* kan ingedeeld worden in twee klassen: horizontale resistentie, gebaseerd op polygenen en verticale resistentie. De verticale resistentie wordt door individuele dominante genen gecontroleerd die *R*-genen genoemd worden. De aanwezigheid van *R*-genen beschermt de plant tegen de schimmel door een overgevoeligheidsreactie. In het verleden zijn 11 *R*-genen geïntroduceerd, die de cultuuraardappel (*Solanum tuberosum*) resistentie geven tegen specifieke fysio's van *P. infestans*. Deze resistentiegenen zijn afkomstig van de hexaploide wilde soort *S. demissum*.

De schimmel toonde van zijn kant het vermogen om gedurende de vegetatieve of generatieve ontwikkelingsfase nieuwe aangepaste verschijningsvormen te ontwikkelen. Dit proces veroorzaakte enerzijds resistentie tegen de fungiciden en anderzijds konden de in de plant aanwezige genetische resistentiefactoren doorbroken worden. Beide aspecten zijn er de oorzaak van dat deze ziekte moeilijk te beheersen is.

Moleculaire isolatie van *R*-genen is zowel van theoretisch als praktisch belang om deze ziekte te kunnen beheersen. Het ontrafelen van het resistentiemechanisme kan mogelijk aanleiding geven tot de ontwikkeling van nieuwe strategieën voor meer duurzame resistentie. Homologie tussen verschillende *R*-genen kan uitmonden in toetsmethoden, indien selectie van veredelingsmateriaal vrij van *R*-genen nodig is, alsmede voor isolatie en gecombineerde toepassing van nieuwe *R*-genen die aanwezig kunnen zijn in niet eerder gebruikte accessies van *S. demissum*. Isolatie van *R*-genen door middel van eiwitisolatie en cDNA klonering is niet mogelijk omdat dit

genprodukt niet bekend is. Een alternatieve benaderingswijze is d.m.v. insertiemutagenese m.b.v. transposons (transposon tagging). Gedurende de laatste 10 jaar is het duidelijk geworden dat bij plantensoorten zoals maïs en leeuwebek door insertiemutagenese met (niet)-autonome transposons plantengenen geïdentificeerd en geïsoleerd kunnen worden. Het *Ac/Ds* systeem van maïs, met het niet-autonome *Ds (Dissociation)*-element en het autonome *Ac (Activator)*-element (die ook het *Ds*-element activeert), kon na moleculaire isolatie met succes m.b.v. transformatie naar andere plantensoorten worden overgebracht. Bij tomaat en aardappel bleek het ingebrachte *Ac*-element actief te zijn terwijl het *Ds*-element door het transposasegen van *Ac* geactiveerd kon worden. Het is bij maïs bekend dat de *Ac/Ds* elementen zich voornamelijk naar nauw gekoppelde posities verplaatsen. Voor de succesvolle aanwending van deze benaderingswijze bij aardappel is het nodig dat de positie van het gewenste *R*-gen op het aardappelgenoom bekend is en dat een transposon-bevattend T-DNA daaraan nauw gekoppeld is (doelgerichte transposon tagging). Het tetraploïde karakter van de aardappel is een ander knelpunt die de ontwikkeling en het gebruik van diploïde aardappelgenotypen voor zowel genetische analyses als "doelgerichte transposon tagging" nodig maakt. Gedurende de laatste 10 jaar is de constructie van genetische kaarten gebaseerd op RFLP (Restrictie Fragment Lengte Polymorfie) merkers ook bij aardappel op gang gekomen. Dit maakt ontdekking van koppeling tussen een *R*-gen en specifieke RFLP merkers op een bepaald chromosoom mogelijk. De kaartpositie van de *R*-genen, hun koppeling of mogelijke allelische relaties waren bij het begin van dit project niet bekend, met uitzondering van het *R1*-gen dat op chromosoom 5 gelokaliseerd was. Het onderzoek dat in dit proefschrift beschreven wordt heeft zowel betrekking op genetisch onderzoek naar en de lokalisatie van verschillende *R*-genen, als ook op de ontwikkeling van resistent plant materiaal dat geschikt is voor transformatie en transposon tagging van betrokken *R*-genen op diploïd niveau. De eerste stap was de reductie van het ploïdieniveau van tetraploïde rassen en testergenotypen die specifieke *R*-genen tot expressie brengen.

Van 10 tetraploïde rassen, die één of meer *R*-genen *(R1, R2, R3, R10)* tot expressie brengen, werden 157 parthenogenetische dihaploïden geïsoleerd met het doel deze genen te lokaliseren (Hoofdstuk 2). De meeste van deze dihaploïden vertoonden morfologische afwijkingen en weinig groeikracht. Zij werden op resistentie tegen *P. infestans* fysio 0 onderzocht om de aan- of afwezigheid van expressie van *R*-genen vast te stellen. Het aantal dihaploïde nakomelingen met resistentie die verkregen werden uit deze *R*-genen bevattende rassen was veel lager dan verwacht mocht worden. Acht van de 60 resistente dihaploïden gaven na kruising zaad. Door middel van

gedetailleerde toetsing van deze 8 klonen op aanwezigheid van resistentie tegen specifieke fysio's konden de fenotypen *R1*, *R3*, *R1R3* en *R1R3R10* aangetoond worden. Eén kloon (Esc.42: afkomstig van cv Escort) bevatte *R1R3* en een andere kloon (Her.64: afkomstig van cv Hertha) bevatte verrassenderwijs naast *R1R3R10* een additioneel *R*-gen dat niet tot expressie gekomen was in het oorspronkelijke ras Hertha. Negentien F_1 nakomelingschappen van de acht resistente dihaploïden werden op expressie van resistentie m.b.v. *P. infestans* fysio 0 onderzocht. Specifieke schimmel isolaten werden later gebruikt om de splitsing van individuele *R*-genen te kunnen analyseren. Een afwijkende splitsing ten gunste van vatbaarheid werd voor het *R3*-gen in 15 van de 17 onderzochte F_1 populaties gevonden, terwijl het *R1*-gen in vijf F_1 populaties naar verwachting splitste. Onafhankelijke splitsing van *R1* en *R3* was zichtbaar in twee F_1 populaties van kruisingen tussen de dihaploïd Esc.42 en vatbare bestuivers. Eén van de twee F_1 populaties, J91-6165, liet een tekort aan *R3* genotypen zien terwijl *R1* normaal splitste. De tweede F_1 populatie J91-6164, die duidelijke splitsing vertoonde voor zowel *R1* als *R3*, werd succesvol gebruikt in RFLP analyses. Koppelingsanalyse tussen *R1*, *R3* en RFLP merkers, gelegen op bekende plaatsen van het aardappelgenoom, bevestigde de plaats van het eerder gelokaliseerde *R1*-gen op chromosoom 5 en positioneerde *R3* op een distale plaats van chromosoom 11.

Bij verder onderzoek naar de positie op het genoom van andere *R*-genen, behalve *R1*, *R3* en *R10*, werden de testerklonen MaR2, MaR4, MaR5, MaR6, MaR7 MaR8, MaR9 en MaR11 gebruikt. Testerklonen, die elk een specifiek *R*-gen tot expressie brengen, zijn in het verleden ontwikkeld om isolaten van *P. infestans* te identificeren. Alleen de tetraploïde testerklonen MaR6 en MaR7, die *R6* of *R7* bevatten, konden succesvol als ouderklonen gebruikt worden (Hoofdstuk 3). Drie dihaploïden werden van MaR6 geïsoleerd. Zij gaven echter geen zaden na kruising met diploïde vatbare bestuivers. Om het probleem van onvoldoende inductie van dihaploïden het hoofd te kunnen bieden werd kloon MaR6 met een vatbare tetraploïde bestuiver gekruist. De F_1 populatie liet na inoculatie met *P. infestans* fysio 0 een 5 : 1 splitsing zien (resistent-vatbaar). Dit resultaat liet duidelijk zien dat *R6* in MaR6 in duplex-vorm aanwezig was. Vijf resistente F_1 nakomelingen werden als ouder voor inductie van dihaploïden geselecteerd en gebruikt. In totaal werden 42 dihaploïden verkregen waarvan er 29 resistent met het *R6*-gen waren. De inductie van voldoende resistente dihaploïden van MaR7 was in één stap mogelijk. Zes dihaploïden werden er geïsoleerd waarvan er twee *R7* tot expressie brachten. Het zelfde fenomeen van lage mannelijke en vrouwelijke fertiliteit, zoals reeds eerder aangegeven, werd ook waargenomen bij de dihaploïden met *R6* of *R7*. Na kruising met vatbare diploïde

testerklonen werden respectievelijk uit zes dihaploïden met *R6* en uit één dihaploïd met *R7* zaden verkregen. In het algemeen werd, zoals eerder bij *R3*, een overmaat van vatbare genotypen in deze F_1 populaties aangetroffen. Aan de andere kant was hier geen aanwijzing voor een veranderde *R*-specificiteit. Van de moleculaire merkers die bij eerdere RFLP analyse met *R1* dan wel met *R3* koppeling lieten zien, vertoonden ditmaal probes die gekoppeld waren met *R3* op chromosoom 11 een duidelijke koppeling met zowel *R6* als *R7*. Het recombinatiegedrag en de recombinatiefrequentie, tussen moleculaire merkers en deze drie *R*-genen, waren niet hetzelfde. Het aantal keer dat recombinatie gevonden werd tussen de RFLP locus en de *R3* of *R7* locus was gering en bovendien steeds in de klasse van vatbare genotypen. In de F_1 nakomelingschap van *R6* werd daarentegen vaker recombinatie gevonden en steeds in de klasse van resistente genotypen. Uit onze waarnemingen wordt niet duidelijk in hoeverre *R3*, *R6* en *R7* allelisch zijn of tot een complex locus behoren.

Transformatie is de eerste stap om het niet-autonome maïs transpositie-element *Ds* bij aardappel binnen te brengen en te komen tot doelgerichte "transposon tagging". Geschikte diploïde aardappelgenotypen moeten ontwikkeld worden waarmee dit onderzoek uitgevoerd kan worden. Koppeling tussen *Ds*-bevattende T-DNA integratie plaatsen en *R*-genen kan alleen ontdekt worden als een groot aantal transformanten beschikbaar is. Dit betekent dat goed transformeerbare diploïde genotypen, die bij voorkeur het betreffende *R*-gen bevatten, geselecteerd moeten worden (Hoofdstuk 4). De competenties voor regeneratie en transformatie werden in 11 resistente diploïden (*R1r1*) onderzocht. De regeneratiepercentages van deze genotypen varieerden tussen de 55% en 95%. Toetsing op transformatie liet zien dat alleen één kloon (J91-6167-2), afkomstig van cv. Hertha, een hoge transformatie-efficiëntie (TE) van 24% bezat. Deze kloon was echter mannelijk steriel. Ook de bes- en zaadzetting waren laag. Om het probleem met fertiliteit het hoofd te kunnen bieden werd deze kloon met een andere vatbare en goed transformeerbare diploide bestuiver (87-1024-2) gekruist. De F_1 nakomelingschap werd getoetst op regeneratie en transformatievermogen als ook op resistentie tegen *P. infestans* fysio 0. Voor transformatie werd *Agrobacterium tumefaciens* stam AM8706 gebruikt die het NPTII-gen voor kanamycine resistentie en het reportergen ß-glucuronidase (GUS) bevat. Deze F_1 populatie van 26 planten splitste voor regeneratievermogen, snelheid van regeneratie en voor regeneratie aan de twee snijoppervlakken van stengelexplantaten. Een paar genotypen liet hogere waarden voor deze eigenschappen zien dan de beide ouders. Splitsing werd ook voor transformatie-efficiëntie gevonden. Eigenschappen als snelle regeneratie en regeneratie aan beide snijoppervlakken van explantaten na transformatie werd ook met selectieve groei op

kanamycine bevattend medium gevonden bij respectievelijk vier en acht genotypen, terwijl deze eigenschappen niet aanwezig waren bij de ouderklonen. Gebaseerd op al deze waarnemingen werd de goed transformeerbare en fertiele kloon J92-6400-A16 geselecteerd. Deze kloon vormde hoofdzakelijk diploïde transformanten en was heterozygoot voor *R1*. In eerder genoemde populatie werd koppeling waargenomen tussen deze competentiefactor voor transformatie enerzijds en het *R1*-gen met een daaraan gekoppelde moleculaire merker op chromosoom 5 anderzijds. Waargenomen werd dat de vrouwelijke fertiliteit bij de transformanten van de geselecteerde kloon als gevolg van transformatie veel minder afnam dan de mannelijke fertiliteit.

De heterozygote *R1r1* kloon J92-6400-A16 werd getransformeerd met het construct *pHPT::Ds*-Kan om een begin te maken met het "taggen" van het *R1*-gen door het *Ds*-transposon van maïs te gebruiken (Hoofdstuk 5). Het T-DNA van het construct bestond uit het *Ds*-element, dat het kanamycine resistentiegen NPTII bevat, dat vervolgens geplaatst werd tussen de promotor en het structurele HPT gen coderend voor hygromycine resistentie. Selectie van transformanten was gebaseerd op kanamycine resistentie. Hygromycine resistentie werd gebruikt om op excisie van het *Ds*-element te selecteren dat na een tweede transformatie geïnduceerd werd door het ingebrachte *Ac*-transposase gen. Inoculatie van 400 stengelexplantaten resulteerde in 377 transformanten waarvan er 65 tetraploïd waren. De 312 diploïde transformanten werden gebruikt voor moleculaire analyse. In 24% van hen was één T-DNA kopie geïntegreerd terwijl in de rest 2-8 kopieën gevonden werd. Uit 187 transformanten werden genomische DNA sequenties die het T-DNA flankeren geïsoleerd d.m.v. IPCR (Inverse Polymerase Chain Reaction) of PR (PLasmid Rescue). Van de 216 geïsoleerde probes bleken er 96 polymorf te zijn en werden er 60 gelocaliseerd. Op basis hiervan kon een gedetailleerde analyse van integratie van T-DNA in het genoom van diploide aardappel worden uitgevoerd. Gebaseerd op de fysische chromosoomlengte en/of het euchromatinegehalte bleken de T-DNA integratie plaatsen volgens willekeur over alle 12 chromosomen verdeeld te zijn. Deze willekeurige verdeling werd ook gevonden als de chromosoomlengte gebaseerd werd op de RFLP kaart. De T-DNA integratie plaatsen van twee transformanten, BET92-Ds-A16-259 en BET92-Ds-A16-416, waren in afstotingsfase met het *R1*-gen gekoppeld. Genetisch analyse van de F_1 nakomelingschap van BET92-Ds-A16-416, na kruising met een vatbare ouder, bevestigde de koppeling tussen de T-DNA integratie plek en het *R1*-gen. Vier recombinanten werden ontdekt met het *Ds*-bevattende T-DNA gekoppeld in koppelingsfase met het *R1* gen. Zij vormen de basis-planten voor vervolgonderzoek naar transposon tagging van het *R1*-gen. Mobilisatie van het *Ds*-element na introductie

van het *Ac*-transposase gen via een tweede transformatie bleek succesvol in 4 verschillende transformanten met één *Ds*-bevattend T-DNA. Met deze nieuwe transformanten zijn geen vervolganalyses uitgevoerd.

Tijdens de ontwikkeling van diploide populaties voor RFLP analyse werd eenmalig een veranderde *R*-specificiteit gevonden en vaak bleek een overmaat aan vatbare planten aanwezig te zijn (Hoofdstuk 2). Het fenomeen van veranderde *R*-specificiteit werd meer gedetailleerd onderzocht in ouderlijk materiaal dat *R4* of *R10* bevatte. Een duidelijke aanwijzing voor de betrokkenheid van additionele genetische factoren werd gevonden door het tot expressie komen van niet eerder in dit materiaal geïdentificeerde *R*-genen (Hoofdstuk 6). De produktie van dihaploïde nakomelingen van de tetraploïde kloon J90-6028, met het *R4*-gen, was niet mogelijk doordat de bloeiperiode te kort was. Zoals eerder aangegeven in Hoofdstuk 3 voor kloon MaR6, kon dit probleem opgelost worden na kruising van J90-6028 met een vatbare tetraploïde bestuiver. Deze tetraploïde F_1 nakomelingschap produceerde een teveel aan vatbare planten maar een verandering van de R-specificiteit werd in de resistente planten niet gevonden. Uit drie tetraploïde F_1 nakomelingen konden ditmaal met *S. phureja* 64 parthenogenetische dihaploïden verkregen worden. In deze groep planten werd na inoculatie met *P. infestans* fysio 0 onverwacht een teveel aan resistente dihaploïden aangetroffen. Als gevolg van de lage fertiliteit van de dihaploïden gaf slechts één plant zaden na kruising met twee vatbare bestuivers. Nader onderzoek naar het resistentiespectrum van deze dihaploïd liet zien dat de *R*-specificiteit veranderd was. In plaats van alleen resistentie tegen het nul-fysio werd ook resistentie tegen fysio 4 gevonden. Drie van de 6 planten uit de eerder genoemde twee F_1 populaties waren ook resistent tegen dezelfde fysio's van *P. infestans*. Echter slechts één ervan (BE93-4031-1) gaf na kruising met een vatbare bestuiver zaden. De F_1 nakomelingschap van BE93-4031-1 bestond uit veel zaailingen en bevatte een overmaat aan vatbare planten. *R*-specificiteitsonderzoek bij 26 resistente planten liet zien dat slechts één ervan *R4*-specifiek was en dat de andere 25 planten nog steeds de veranderde *R*-specificiteit vertoonden. Kruising met de *R4*-specifieke resistente plant was helaas niet mogelijk. Kruising met één van de andere 25 planten met de veranderde *R*-specificiteit was wél mogelijk. De F_1 populatie liet met het nul-fysio van *P. infestans* een duidelijke 1:1 splitsing zien terwijl daarnaast geen *R4*-specifieke planten gevonden werden.

Zestien parthenogenetische dihaploïden van cv. Primeri met het *R10*-gen vertoonden de verwachte 1:1 splitsing. Fysio specifieke analyses lieten alleen expressie van het *R10*-gen in de resistente dihaploïden zien. In splitsende F_1 populaties,

afkomstig van twee van deze resistente dihaploïden, werd bij alle resistente planten een veranderde fysio-specificiteit gevonden (resistent tegen fysio 4.10). Dit geeft duidelijk de aanwezigheid aan van een niet-geïdentificeerd *R*-gen. Na een tweede kruisingscyclus werd in één van de families een duidelijke splitsing voor fysio-specificiteit gevonden. Zeven van de 21 resistente planten waren vatbaar voor fysio 4.10 en daarmee *R10*-specifiek. Dit fenomeen van veranderende *R*-specificiteit kan verklaard worden door het bestaan en de splitsing van een tweede genetische factor die de expressie van niet-geïdentificeerde *R*-genen beïnvloedt.

De aanwezigheid van zo'n tweede genetische factor die de expressie van een bekend *R*-gen beïnvloedt werd in verband met *R1* onderzocht. Tien vatbare "recombinanten" die nog steeds het aan resistentie gekoppelde RFLP allel van probe *GP21* bevatten werden gekruist met een vatbare bestuiver. Vier van deze geselecteerde planten gaven zaden. Genotypen resistent tegen *P. infestans* fysio 0 werden in drie van de vier F_1 nakomelingschappen gevonden. Zoals verwacht, liet nader onderzoek van de resistente planten expressie van het *R1*-gen zien. Dit is een duidelijke aanwijzing dat het *R1*-allel niet tot expressie kwam in de ouderklonen als gevolg van de aanwezigheid van een "suppressor" of als resultaat van complementaire gen werking. Verder onderzoek is nodig om tot lokalisatie en identificatie van de aard van deze onbekende factor te komen.

Op basis van de beschreven resultaten is het duidelijk dat de *R*-genen in diploïd plant materiaal gelokaliseerd en "getagged" kunnen worden. De *R3, R6* en *R7* allelen kunnen deel uitmaken van een complex locus, allelisch zijn of behoren tot verschillende gekoppelde loci. De ontdekking van de tweede genetische factor die bij de expressie van *R*-genen betrokken is bemoeilijkt bij aardappel het genetisch onderzoek aan resistentie tegen *P. infestans* en de praktische veredeling. De selectie van een goed transformeerbare *R1r1* diploïd maakte de ontwikkeling van honderden transformanten met een *Ds*-bevattend T-DNA mogelijk. De 60 transformanten met een gelokaliseerde *Ds*-bevattend T-DNA insert verdeeld over het gehele aardappelgenoom kunnen niet alleen gebruikt worden bij de isolatie van *R*-genen maar ook van genen coderend voor andere eigenschappen. De competentie voor transformatie kon eenvoudig naar andere diploide genotypen overgebracht worden.

Curriculum Vitae

Ali El-Kharbotly was born on 12 May, 1961 in Cairo Egypt. In June 1978, he obtained the Secondary school diploma and started his academic study. He got the B.Sc. degree in Horticultural Science in June 1982 from the Faculty of Agriculture, Ain Shams University, Cairo, Egypt. He joined the Military Service until January 1984 as an officer in the air defence system. In 1984 he started his academic carrier as a demonstrator at the Department of Horticulture, Ain Shams University, Cairo. In the mean time, he attended several post graduate courses. In October 1986, he joined the post graduate programme at the Mediterranean Agronomic Institute of Chania, Crete, Greece, where he obtained a post graduate diploma in Vegetable Seed Production in 1987, followed by the M.Sc. Degree in Subtropical Crops and Protected Cultivations in 1989 from the same Institute. In 1990, he carried out a fertilization experiment on rice at the Research Station for Rice, Kogoni, Mali.

Since 1991, he worked voluntary as a researcher at the Department of Plant Breeding of Wageningen Agricultural University and at the Center for Plant Breeding and Reproduction Research (CPRO-DLO), under the supervision of Prof. Dr. Ir. E. Jacobsen and Dr. A. Pereira. The result of this research is documented in this thesis to fulfil the Ph.D. degree requirement at the WAU. Starting from 15 October 1995, he will work as a lecturer and researcher at the Department of Plant Science, Faculty of Agriculture, Sultan Qaboos University, Oman.