Identification of potato genes involved in *Phytophthora infestans* resistance by transposon mutagenesis

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Stellingen

1. De transpositie activiteit van het autonome maïs transposon *Ac* in aardappel wordt bepaald door de genoom positie.

   Peterson, P.A. (1977) The position hypothesis for controlling elements in maize. In *DNA insertion elements, plasmids and episomes* (Bukhari, A.I., Shapiro, J.A. and Adhya, S., eds). New York: Cold Spring Harbor laboratory, pp. 429-435.

   Dit proefschrift.

2. De resistentie reactie geïnduceerd door een dominant *R* gen is altijd afhankelijk van de expressie van andere genen.

   Dit proefschrift

3. Elk gen is een QTL.

4. Nu we met het beschikbaar komen van de humane DNA-sequentie het "boek des levens" grotendeels in handen hebben, zullen we opnieuw moeten leren lezen.

5. Transposons zijn er omdat ze in staat zijn zichzelf te vermenigvuldigen, het lijken wat dat betreft wel mensen.

   Vrij naar Ronald Plasterk, Het nut van junk-DNA, Intermediair, 17 september 1998.

6. Elke boer is een biologisch ondernemer, ook de biologische boer.

7. RSI is een gevolg van werkhouding.

8. Rondom DNA bestaan veel misverstanden.

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L. J. G. van Enckevort

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Bibliografic abstract Potato genes acting in the *R1* type HR resistance reaction upon infection with *Phytophthora infestans* were identified using a transposon tagging strategy in diploidised potato. Somatic and germinal *Ac* and *Ds* transposition was characterised both phenotypically and molecularly. Protoplast isolation and cell specific selection for *Ds* excision enabled the direct selection of somatic excision events, resulting in the regeneration of a potato transposon tagged population with predominantly new independent *Ds* mutations. Inoculation with *P. infestans* race 0 and quantification of the HR resistance response identified four putative transposon tagged mutants that showed a distinct altered *R1* resistance response. Sequence analysis on the *Ds* insertions in one mutant identified significant homology to receptor kinase-like proteins. In total 11 different *Solanum tuberosum* protein kinase (StPK) homologs were isolated and the transposon mutated StPKs were designated *rpr1* and *rpr2*, genes required for *P. infestans R1* resistance.

Key words *Solanum tuberosum, Phytophthora infestans, Ac/Ds* transposon tagging, somatic selection, *R* gene mediated resistance

In herinnering aan mijn vader

Contents

Chapter 1

General introduction

Potato and *Phytophthora infestans*

Potato is one of the world's most important food crops, exceeded only by maize, rice and wheat in total production (FAO, 2000). In The Netherlands, potato is the largest food crop with a total production of 8,200,000 metric ton in 1999 (FAO, 2000). The most serious disease affecting a potato crop during the growing season is late blight, in Dutch 'de aardappelziekte'. Destruction of foliage and stems, particularly under moist and warm weather conditions, results in serious harvest damage, which is followed by a decay of tubers while in storage.

The first outbreak of late blight in Europe was reported by the end of June 1845 in Belgium and extended all over Europe during that season (Bourke, 1964). Losses in the potato crop were most severe in Belgium, The Netherlands and northeast of France. In 1846 the effect of the late blight attack was most dramatic in Ireland where in early August the crop was destroyed in most areas and the year which followed marked an intense period of great hunger, the Irish Potato Famine (Salaman, 1949). By 1851 the population of Ireland had decreased by 2,5 million people due to starvation, disease and emigration.

The fungus causing this devastating potato disease was named by de Bary in 1876 *Phytophthora infestans* (Mont.) (Bary, 1876). The genus *Phytophthora* is classified in the family Pythiaceae and belongs to the class oomycetes, order Peronosporales. Oomycetes have many fungus-like characteristics but biochemical and phylogenetic analyses of ribosomal and mitochondrial gene sequences suggest that oomycetes be more closely related to golden-brown algae and heterokont algae in the Kingdom Protista (Kumar and Rzhetsky, 1996; Paquin *et al.*, 1997; Peer and de Wachter, 1997).

Nowadays *P. infestans* is still worldwide the main pathogen of the potato crop. Detection of the A2 mating type in Europe in the early 1980s indicated that the population composition of *P. infestans* was changing. Also in The Netherlands the *P. infestans* populations were much more diverse then those prevailing before 1980 (Drenth *et al.*, 1993; 1994). The displacement of old populations by genotypically new ones and subsequent migrations in the 1980s and 1990s resulted in increasingly diverse *P. infestans* populations all over the world (Fry *et al.*, 1993; 1992; Goodwin, 1997). In The Netherlands an increase in the severity of potato late blight epidemics is observed since 1979 (Zwankhuizen and Zadoks, 1998).

Chemical control by application of fungicides is still the most important measure for controlling a potato late blight epidemic. This generates environmental concern and also contributes to the emergence of resistant isolates (Davidse *et al.*, 1981). In the short term, a reduction of the volume of fungicides used can be achieved by increased efficacy of compounds (Egan *et al.*, 1995) and, to a lesser extent, by substitution of calendar spraying with supervised control via decision support systems (Schepers *et al.*, 1995). In the long term, resistant potato cultivars should reduce the dependence on fungicides.

Resistance to *Phytophthora infestans*

Disease development of *P. infestans* in potato is well studied and characterised (Coffey and Gees, 1991). *P. infestans* enters the plant by penetration of an epidermal cell. Branching hyphae expand from this site of penetration through the intercellular space and feeding structures, known as haustoria, enter neighbouring cells. In this way the hyphae colonises the leaf, finally resulting in sporulation. These spores can be the start of a late blight epidemic in the field when weather conditions are favourable.

In a resistant potato genotype the infection process is blocked at an early stage due to recognition of the pathogen and subsequent activation of host defence responses. Potato breeding for resistance to *P. infestans* in the first decades of the 20th century resulted in the identification of *P. infestans* resistance genes from the Mexican wild species *Solanum demissum* (Black *et al.*, 1953; Mastenbroek, 1952) and *S. stoloniferum* (McKee, 1962). Segregation analysis among tetraploid potato cultivars demonstrated the Mendelian inheritance of these resistances as single dominant factors (Malcolmson and Black, 1966; Mastenbroek, 1952). In total 11 *R* (resistance) genes were identified in the hexaploid wild species *Solanum demissum* and introgressed in tetraploid potato cultivars (Malcolmson, 1969; Umaerus *et al.*, 1983; Wastie, 1991).

In *R* gene resistant potato genotypes with high resistance levels, a group of cells around the first site of attempted infection display the hypersensitive response (HR) leading to local cell death causing necrotic spots and stopping the *P. infestans* infection. This activation of HR is highly specific and is induced in a specific genotype upon recognition of a specific race of the pathogen. Many plant species use the HR

response to defend themselves against infection by viruses, bacteria, fungi, oomycetes, nematodes, insects, and even other plants. The mechanism is characterised by the gene-for-gene relationship that requires a specific resistance (*R*) gene from the plant and a corresponding avirulence (*Avr*) gene from the pathogen (Flor, 1942; Hammond-Kosack and Jones, 1997; Keen, 1990). Without these genes plant defences are not quickly activated and infection by the pathogen is permitted. *R* genes in most species do not provide durable resistance because of the rapid evolution of new virulent races of the pathogen. Plants and pathogens can harbour a number of corresponding pairs of resistance and avirulence genes that interact to provide the signals for induction of disease resistance (Keen, 1990). To understand how specific plant defence is regulated, it is necessary to learn the nature of the *R* and *Avr* gene products, the way they interact, and the chain of events that result.

Due to the rapid occurrence of virulent races of *P. infestans*, *R* genes as a source of resistance have been abandoned and breeding efforts started to concentrate on the increase of unspecific resistance to *P. infestans* which seems to be polygenically inherited (Umaerus *et al.*, 1983; Wastie, 1991) and is found in *S. demissum* and in other wild *Solanum* species (Colon and Budding, 1988; Toxopeus, 1964). This horizontal resistance to *P. infestans* is, however, methodologically difficult to test and frequently the resistance reaction cannot be separated from the effect of major *R* genes present in the same genetic background (Colon and Budding, 1988; Gees and Hohl, 1988; Graham, 1963; Swiezynski *et al.*, 1991).

Genetic mapping

Since the first maize map in 1935 (Emerson *et al.*, 1935), maps of morphological characters and later isozymes have been published, however these have often limited numbers of characters per linkage group. With the development of the DNA based methods RFLP (restriction fragment length polymorphisms; Botstein *et al.*, 1980), RAPD (randomly amplified polymorphic DNA; Williams *et al.*, 1990) and AFLP (amplified fragment length polymorphism; Vos *et al.*, 1995) detailed linkage maps have been developed for a number of species, including potato.

The tetrasomic inheritance of the potato crop prevented the development of a classical genetic map of potato. Dihaploids made genetic analysis more

straightforward, although male sterility and self-incompatibility prevented the construction of pure potato lines. Therefore, the first molecular marker maps of potato were derived from highly heterozygous diploid parents (Bonierbale *et al.*, 1988; Gebhardt *et al.*, 1991; 1989). The use of molecular markers in diploid populations, segregating for specific resistances to nematodes, viruses and oomycetes, enabled the localisation of several resistance genes on the potato genome.

The top arm of chromosome 5 has been shown to contain a number of resistance loci including *Rx2* conferring extreme resistance to most strains of the virus PVX (Ritter *et al.*, 1991); the *P. infestans* resistance gene *R1* (Leonards-Schippers *et al.*, 1992); *Gpa* giving resistance to the nematode *Globodera pallida* (Kreike *et al.*, 1994); *Nb* conferring hypersensitive resistance against the virus PVX (Jong *et al.*, 1997) and *Grp1* contributing major resistance to a *G. rostochiensis* strain and partial resistance to a *G. pallida* strain (Rouppe van der Voort *et al.*, 1998). In this same chromosome V region a homolog of the tomato *Pseudomonas syringae* pv. *tomato* resistance gene *Pto* was identified by PCR analysis (Leister *et al.*, 1996). QTL mapping in *S. tuberosum* lines identified strong effects on *P. infestans* foliage resistance (Leonards-Schippers *et al.*, 1994), tuber resistance, foliage maturity, vigour (Collins *et al.*, 1999; Oberhagemann *et al.*, 1999) and tuberization (Berg *et al.*, 1996) in this chromosome 5 region. These QTLs very likely represent minor genes in many genotypes that play a role in both *P. infestans* resistance and developmental processes which indirectly influence the resistance response. This potato genomic region is therefore, one of the most interesting chromosomal locations for studying the evolution of resistance gene mechanisms in the Solanaceae.

R gene isolation

Over the last few years a large number of resistance genes (reviewed in Hammond-Kosack and Jones, 1997) have been isolated. In the complex genome of maize, the *HM1* gene, which confers resistance to the fungal pathogen *Cochliobolus carbonum* race 1 was first cloned by transposon-induced mutagenesis (Johal and Briggs, 1992). The availability of a high density RFLP map (Tanksley *et al.*, 1992) combined with an indirect resistance screen and the availability of near isogenic lines made tomato amenable to positional cloning. The *Pto1* gene, conferring resistance to *avrPto* races

of *P. syringae* pv. *tomato*, was the first resistance gene to be isolated using a map-based-cloning strategy (Martin *et al.*, 1993). Genetic mapping in Arabidopsis combined with the availability of a mutant phenotype enabled the localisation and map-based-cloning of *RPS2*, conferring resistance to *P. syringae avrRpt2* (Bent *et al.*, 1994). The isolation of resistance genes was continued by the application of the maize transposable elements *Ac* or *Ds* combined with positive selection schemes for the tagging of the mapped fungal resistance gene *Cf-9* in tomato (Jones *et al.*, 1994) and the viral resistance gene *N* in tobacco (Whitham *et al.*, 1994). In flax, using a linked *Ac* (29 cM), one mutant was selected which facilitated the isolation of the *L6* resistance gene (Lawrence *et al.*, 1995). In subsequent years advances in map-based-cloning strategies using high density linkage maps in rice, tomato and Arabidopsis have resulted in the isolation of almost 20 resistance genes to date.

The development of high-density linkage maps in potato was stimulated by the availability of the AFLP technique (Eck *et al.*, 1995; Vos *et al.*, 1995). The generation of many closely linked markers is a prerequisite for accurate map-based cloning strategies. The potato virus X resistance gene *Rx1* (Bendahmane *et al.*, 1999) and the *G. pallida* resistance gene *Gpa2* (Vossen *et al.*, 2000), both located and linked in a cluster on chromosome 12, are the first potato resistance genes isolated using a map-based-cloning approach.

Among the isolated *R* genes, 5 classes are identified based on common characteristics that include the nucleotide binding site, leucine-rich repeats, a transmembrane domain and a serine/threonine protein kinase domain (Bent, 1996; Ellis and Jones, 1998; Hammond-Kosack and Jones, 1997). Genetic mapping of resistance gene specificities has indicated that they frequently cluster at complex loci and cosegregate with different resistance specificities (Jia *et al.*, 1997; Leister *et al.*, 1996; Ori *et al.*, 1997; Pan *et al.*, 2000; Parniske *et al.*, 1997; Song *et al.*, 1997). The shared motifs among R proteins identified in different plant species and for different pathogen types, suggest conservation in controlling strain-specific pathogen resistance in plants by similar signalling mechanisms. Despite these significant insights into *R* gene structure, much remains to be elucidated about the mechanisms by which R proteins recognise pathogens and transduce this information in the plant cell to initiate defence responses.

On the basis of the gene-for-gene model, *R* genes were suggested to encode receptors specific for the corresponding *Avr* gene product. One structural motif shared

by almost all isolated resistance genes is the leucine-rich repeat (LRR), which is found to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994; Leckie *et al.*, 1999). It has been postulated that the product of an avirulance gene directly interacts with the LRR region, triggering a pathway that eventually leads to HR. Recent evidence for an *in planta* interaction was found for the NBS-LRR R protein RPS2 and the Avr protein AvrRpt2 in Arabidopsis protoplasts (Leister and Katagiri, 2000).

Receptor-like protein kinases identified in tomato (Pto and Pti; Martin *et al.*, 1993; Tang *et al.*, 1996; Zhou *et al.*, 1995) and rice (XA21; Song *et al.*, 1995) probably play an important role in activating the downstream signalling by phosphorylation after recognition between the R protein and the Avr product. While this interaction seems to be highly specific, in several species it is shown that *R* gene mediated HR resistance responses are not always complete, indicating that other genes play an additional role in their expression levels (El-Kharbotly *et al.*, 1996b; Hammond-Kosack and Jones, 1994; Ordoñez *et al.*, 1997).

Maize transposable elements

A transposable element is a piece of DNA that can move around the genome and is recognised phenotypically when it inserts into a gene and causes a visible mutation. The characteristic property of a transposable element mutation is somatic instability displayed as variegation or chimerism for wild type and mutant sectors. This feature is common to the class of active transposable elements that transpose via a DNA intermediate, by a cut-and-paste mechanism.

McClintock's genetic studies in maize (*Zea mays*) resulted in the initial discovery of elements that caused chromosome breakage displayed by the loss of visual genetic markers (McClintock, 1948; 1950). The site of chromosome breakage was termed *Dissociation* (*Ds*), and the element inducing the breakage events *in trans* was called the *Activator* (*Ac*) (McClintock, 1947). The critical discovery was that the site of breakage (*Ds*) could move (transpose) to another position, resulting in gene insertion and disruption of function. This could be restored by *Ac* element mediated excision of the *Ds* element from the disrupted gene (McClintock, 1951). The molecular isolation and characterisation of the maize *Ac-Ds* elements supplied the evidence that the autonomous *Ac* encodes an element specific transposase protein (Fedoroff *et al.*,

1983). Members of the *Ac-Ds* family contain a specific 11 bp terminal inverted repeat (TIR) and create an 8 bp target site duplication (TSD) on insertion. The 4.565 kb long *Ac* element produces a 3.5 kb mRNA which encodes an 807 amino acid putative transposase (Kunze *et al.*, 1987; Pohlman *et al.*, 1984). This protein has been shown *in vitro* to bind to subterminal sequences at the termini, which are essential for transposition (Kunze and Starlinger, 1989).

Different endogenous transposable element systems are also found in maize (Peterson, 1987) as well as in *Antirrhinum majus* (snapdragon; Coen *et al.*, 1989) and *Petunia hybrida* (Gerats *et al.*, 1990) and in at least 35 other plant species (Nevers *et al.*, 1986). In fact all living organisms probably contain transposable elements at some time in their history but they have not all been genetically characterised as in maize. In some cases these elements are not mobile at present, but are recognised only by the characteristic structural features of transposable elements (Flavell *et al.*, 1994).

Transposon tagging strategies for gene isolation

Transposon tagging enables the isolation of genes based on mutant phenotypes and does not require prior knowledge of the gene's primary function. A mutant allele which is genetically `tagged' with the transposon, can be molecularly isolated by homology to the cloned transposon-tag sequences. DNA sequence information of the transposons can also be used to design primers to isolate the flanking DNA of the tagged gene by PCR. The adjacent gene sequence of the cloned mutant allele is then used to isolate the corresponding wild type gene (Pereira, 1998; Walbot, 1992). This technique is particularly suited for the isolation of genes, whose product is unknown or produced in too little quantities to permit analysis. The scope of transposon tagging is limited by the fact that not all genes display a mutant phenotype when inactivated. This situation can be remedied by the use of heterologous transposons which are modified to function as gene detectors, without necessarily causing a mutant phenotype (Pereira, 1998).

The molecular isolation and characterisation of the maize transposable element *Ac* directly lead to the isolation of the *Bz* gene that was 'tagged' by this element (Fedoroff *et al.*, 1984). Transposon tagging experiments in maize with specific autonomous elements yielded tagged mutants at frequencies of 10^{-5} to 10^{-6} (Döring, 1989). Efforts to increase mutation frequencies at target loci originally made use of

well-characterised high transposon lines. Studies on the transpositional behaviour of the *Ac* elements revealed that the transposed elements reinserted predominantly at positions linked to the original donor site. This information led to the development of strategies (Walbot, 1992) for the use of autonomous elements close to the target locus for tagging, and consequently to high tagging efficiency leading to the isolation of genes involved in plant phenotypes.

In plant species lacking well-characterised transposons, the transposable elements *Ac-Ds* (Baker *et al.*, 1986), *En-I(Spm)* (Masson and Fedoroff, 1989; Pereira and Saedler, 1989), *Tam3* (Martin *et al.*, 1989) and *Tnt1* (Lucas *et al.*, 1995) have been shown to be functional. To monitor excision of transposable elements in heterologous plant species, phenotypic assays were developed using selectable (antibiotic resistance) or visual marker genes (reviewed in Haring *et al.*, 1991; Pereira, 1998). In a typical construct the transposon is inserted in the 5' untranslated leader of a marker gene, blocking its expression. Excision of the element in the plant restores the activity of the marker gene, which can be visualised as resistance to the particular antibiotic. Reinsertion of a transposed element can be selected for when the non-autonomous element contains a marker gene (Masterson *et al.*, 1989). Thus the use of a combination of markers for excision and examination of the element presence reveals that the complete process of transposition has taken place.

The use of transposition markers enabled the comparison of the germinal excision frequency (Jones *et al.*, 1989; Schmidt and Willmitzer, 1989) i.e. the fraction of seedlings displaying excision events, inherited through the germ line and excluding somatic events, among the total number of seedlings in the progeny of a plant. Combined with molecular evidence it was also shown that most properties and features of transposition in heterologous species were found similar to that in maize, e. g. target site duplications and excision footprints. The behaviour of *Ac* to transpose to positions that are closely linked to the original donor site has now been observed in several heterologous species (reviewed in Pereira, 1998). An interesting deviation is found in the excision rate of *Ac*, which shows a negative dosage effect in maize (e.g. 1 *Ac* element displays twice the variegation as 2 *Ac* elements), while in heterologous plants a positive dosage is evident (Hehl and Baker, 1990; Jones *et al.*, 1989).

Excision markers enabled the efficient selection for plants that had undergone transposition events. After selfing, distinct criteria for mutant designation allowed the identification of mutant phenotypes in Arabidopsis (Aarts *et al.*, 1993; Bancroft *et al.*,

1993; Long *et al.*, 1993) and petunia (Chuck *et al.*, 1993). This demonstrated the effectiveness of random or non-targeted transposon tagging experiments of maize transposable elements for the identification of mutants in heterologous species and enabled the subsequent isolation of the corresponding genes. Pre-selected target genes that were only known by their phenotype were also efficiently isolated, e. g. the tomato *Cf-9* gene (Jones *et al.*, 1994), the Arabidopsis *FAE1* gene (James *et al.*, 1995), the *L6* resistance gene from flax (Lawrence *et al.*, 1995) and the tobacco *N* gene (Whitham *et al.*, 1994). The effectiveness of these targeted transposon tagging procedures depended on the activity of the transposons and the genetic distances of the transposons from the target genes. In addition, positive selection strategies in tobacco and tomato facilitated the most direct selection of a mutant phenotype.

Some of the problems associated with heterologous tagging systems have been initially approached by the use of appropriate promoters for expression of the transposase genes (reviewed in Pereira, 1998). The low transcription levels of wild-type transposase genes have been remedied by using the strong CaMV 35S promoter. This increased the excision and transposition rates to useful proportions (Aarts *et al.*, 1993; Honma *et al.*, 1993) but, studies in tobacco showed that a high level of constitutive transposase expression for *Ac*, induced only early excisions and was inhibitory for late transpositions (Scofield *et al.*, 1993). A variety of promoters have been experimented with to control transposition, enabling the creation of a large number of independent insertions for different tagging purposes.

Mutants tagged with transposable elements that are still active may display a phenotype characterised by variegation or chimeric wild-type and mutant clonal sectors. This chimerism is often the first indication for a tagged mutant when it displays a cell-autonomous mutant phenotype. Co-segregation of the mutant phenotype and the specific element is the next step to test (Walbot, 1992). This is best done by DNA blot analysis using first the transposon and then the putative gene after isolation as hybridisation probes. A transposon flanking DNA fragment can be obtained by Inverse PCR (IPCR; Earp *et al.*, 1990), or plasmid rescue if the transposon contains sequences for maintenance and selection in bacteria (Rommens *et al.*, 1992b). Presence of a marker gene within the transposon can aid analysis when no other transposon copies are present in the genome. Later, primers from the putative gene sequences can be used, which in combination with transposon primers, will help distinguish the different alleles in segregation experiments. These primers enable also

the recovery of the wild-type gene sequence from a genomic or cDNA library. To prove that a wild-type allele has been reconstructed and that it can correct the mutant phenotype, a complementation test by transformation is required.

Identification of potato genes involved in *Phytophthora infestans* resistance by transposon mutagenesis, outline of the thesis

To identify genes acting in the *R1* type HR resistance reaction in potato upon infection with a virulent *P. infestans* race, a transposon tagging strategy in diploidised potato was developed. Chapter 2 describes the selection of suitable populations for efficient transposon tagging strategies in potato. The frequencies and patterns of *Ac* and *Ds* transposition, both somatically and germinally, were phenotypically and molecularly characterised (Enckevort *et al.*, 2000b). Using a visual marker, the granule-bound starch synthase (*GBSS*) gene that is responsible for the production of amylose in starch containing organs like tubers, enabled the study of *Ac* excision by monitoring *GBSS* transgene expression in an *amylose-free* (*amf*) potato clone.

The selection of diploid potato clones homozygous for the *R1* resistance gene (R1R1) was hindered by the heterozygosity for deleterious alleles and self-incompatibility. Several generations of crossing and selection were needed to produce vigorous R1R1 plants which showed simultaneously high transposition frequencies for mutant selection, R1Ds/R1-;Ac/- (unpublished). Therefore, an alternative and quicker method was developed to employ directed transposon tagging in diploid potato clones that were heterozygous for *R1* (R1r1).

Chapter 3 describes the selection of diploid R1r1 potato plants with a linked *Ds* and active transposition, R1Ds/r1-;Ac/- (Enckevort *et al.*, 2000a). Instead of searching for germinal transmission, direct selection of somatic excision events was performed in these highly chimaeric plants. Protoplast isolation and the use of hygromycin as a cell specific selection marker for *Ds* excision enabled the direct regeneration of potato plants with new independent *Ds* insertions. Molecular analysis confirmed that predominantly independent *Ds* mutations were selected among the 2000 protoplast regenerants. This approach showed that the somatic selection for *Ds* excision and probably independent transposition could facilitate the production of large tagging populations from R1r1 potato.

The somatically selected protoplast regenerants, R1Ds/r1-;Ac/- with predominantly new independent *Ds* insertions, were inoculated with *P. infestans* race 0 and this first screening identified 33 plants with and altered *P. infestans R1* resistance response (Chapter 4). The detached leaf assay with stringent inoculation conditions on replicate samples enabled quantification of the *R1* type HR resistance response for the parental seedlings and the 33 putative resistance variants. This enabled the identification of four plants, putative transposon tagged mutants, with a distinctly altered *R1* resistance response. Preliminary sequence analysis on the *Ds* insertions in these putative mutants, identified for the tagged genes in mutant 1000 significant homology to receptor kinase-like proteins and for mutant 994 a striking homology to *cis*-responsive regions of defence related genes. So, the screening of the *Ds* transposon-mutagenised population was validated by the identification of these potential HR signalling mutants.

Further molecular analyses were performed to characterise the two *Ds* insertion loci in mutant 1000 (Chapter 5). The *Ds* flanking sequences both showed high homology to the serine/threonine protein kinase domain of the rice *Xanthomonas* resistance gene *Xa21* including all conserved domains and a conserved intron position. In different potato clones a total of 11 different *Solanum tuberosum* protein kinase (StPK) homologs were identified independent of the *R1* locus. The observed *P. infestans R1* resistance mutation is very likely due to the *Ds* transposon insertions in one or both StPKs. The transposon mutated StPKs were designated *rpr1* and *rpr2*, required for *P. infestans R1* resistance.

This thesis concludes with a general discussion (Chapter 6) addressing: 'Transposon tagging strategies for the highly heterozygous diploid potato'; '*R* gene signalling'; and 'Semi-dominant mutations due to transposon insertions'.

Chapter 2

Development of *Ac* and *Ds* transposon tagging lines for gene isolation in diploid potato

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Key words *Ac/Ds* transposition, *GBSS* excision assay, germinal transmission, *Solanum tuberosum*

Abstract

For the development of an efficient transposon tagging strategy it is important to generate populations of plants containing unique independent transposon insertions that will mutate genes of interest. To develop such a transposon system in diploid potato the behavior of the autonomous maize transposable element *Ac* and the mobile *Ds* element was studied. A *GBSS* (*Waxy*) excision assay developed for *Ac* was used to monitor excision in somatic starch-forming tissue like tubers and pollen. Excision of *Ac* results in production of amylose starch that stains blue with iodine. The frequency and patterns of blue staining starch granules on tuber slices enabled the identification of transformants with different *Ac* activity. After excision the *GBSS* complementation was usually not complete, probably due to the segment of DNA flanking *Ac* that is left behind in the *GBSS* gene. Molecular and phenotypic analysis of 40 primary transformants classified into 4 phenotypic classes revealed reproducible patterns. A very high percentage (32.5%) of the primary transformants clearly showed early excision in the first transformed cell as displayed both by the analysis of the *GBSS* excision marker gene as well as DNA blot analyses. Genotypes useful for tagging strategies were used for crosses and the frequency of independent germinal transpositions was assessed. In crosses to *Ds* genotypes, excision of *Ds* was revealed that correlated to the activity of the *Ac* genotype. A line displaying *Ac* amplification to multiple copies conferred a high frequency of independent *Ds* transpositions. The genotypes described here are useful in somatic insertion mutagenesis aimed at the isolation of tagged mutations in diploid potato.

Introduction

Transposable elements are useful molecular genetic tools to mutate and identify genes. The transposable elements *Ac* and *Ds* were first characterised in maize (McClintock, 1950) and their molecular isolation led to the identification of maize genes that were tagged by these elements (Fedoroff *et al.*, 1983). The *Ac* element is able to transpose autonomously and also to induce transposition of the non-autonomous *Ds* elements that are transposase defective. Introduction of these elements in heterologous species demonstrated their utility for isolating genes in self-fertilising plant species such as petunia (Chuck *et al.*, 1993), Arabidopsis (Bancroft *et al.*, 1993), tobacco (Whitham *et al.*, 1994), tomato (Jones *et al.*, 1994) and flax (Lawrence *et al.*, 1995). Also in the highly heterozygous and tetraploid potato, *Ac* and *Ds* were shown to be functional in diploid genotypes that are essential for tagging (Enckevort *et al.*, 2000a; Chapter 3; Knapp *et al.*, 1988; Pereira *et al.*, 1991). Tetraploid potato can be diploidised but becomes self-incompatible (Cipar, 1964; Jong and Rowe, 1971) and therefore, stays highly heterozygous in subsequent generations. In comparison to a self-fertilising crop like tomato, the selection of homozygous potato clones needed for the production of efficient transposon tagging populations is hindered by heterozygosity for deleterious alleles and self-incompatibility. Several generations of crossing and selection are needed to produce tagging lines, which are homozygous for the gene of interest and show high transposition frequencies for mutant selection.

To develop suitable populations for efficient transposon-tagging strategies in potato it is important to know from selected lines the frequencies and patterns of *Ac* and *Ds* transposition, both somatically and germinally. The occurrence and frequency of transposition events can be studied using visual markers like streptomycin resistance (Jones *et al.*, 1989), ß-glucuronidase (*GUS*; Finnegan *et al.*, 1989), *rolC* (Spena *et al.*, 1989) and *Lc* (Goldsbrough *et al.*, 1996). These cell specific marker genes enable the detection of transposition during plant development. In potato another visual marker is available, the granule-bound starch synthase (*GBSS*) gene that is responsible for the production of amylose in starch containing organs like tubers (Leij *et al.*, 1991). Normal wildtype potato starch consists of approximately 20% amylose and 80% amylopectin. The cell specific *GBSS* gene expression pattern can easily and accurately be determined by iodine staining of starch granules. Low expression levels of the *GBSS* gene results in less or no formation of amylose and the

starch granules stain red with iodine. Starch granules with some expression of the *GBSS* gene, resulting in low amounts of amylose, display a blue staining central core surrounded by red staining growth rings. Starch granules that contain full *GBSS* gene expression, resulting in wildtype levels of amylose, stain completely blue (Kuipers *et al.*, 1994). With these visual differences in amylose formation in starch granules it is possible to phenotypically semi-quantify *GBSS* gene expression.

By introducing a *GBSS* transgene into a potato clone that was mutated for the *GBSS* gene (Hovenkamp-Hermelink *et al.*, 1987), frequent full complementation, based on iodine staining and restored *GBSS* gene activity was documented (Flipse *et al.*, 1994). For monitoring *Ac* excision in potato an autonomous *Ac* element including about 60-bp of flanking DNA from the maize *Waxy* gene (Behrens *et al.*, 1984) was inserted between the transcription and translation starts of a genomic *GBSS* clone. Introduction of this *Ac GBSS* transgene in an *amylose-free* (*amf*) potato clone enabled the study of *Ac* excision by monitoring *GBSS* transgene expression using iodine staining of starch tissue. Preliminary data on the use of the *GBSS* gene as a visual marker for displaying *Ac* excision in potato starch tissue has been reported (Pereira *et al.*, 1991). The present paper describes the further phenotypic and molecular characterisation of the somatic *Ac* excision patterns of 40 *Ac* T-DNA transformants and adds information on T-DNA copy number, *Ac* excision and transposition frequencies. The *GBSS* gene expression visualised in individual starch granules of the primary *Ac GBSS* transformants and the *Ac* excision pattern is discussed in relation to these data.

Transposition events that pass through meiosis and are inherited in the sexual progeny of a plant are referred to as germinally transmitted transposition events. The germinal transmission frequency of transposition events determines the number of potentially useful mutagenic events in progeny seedlings. In developing an effective two-element transposon tagging strategy knowledge on the germinal transmission frequency of non-autonomous *Ds* elements, induced by an autonomous *Ac* element, is important. The observed levels of autonomous *Ac* and induced *Ds* transposon frequencies are discussed.

Materials and methods

Plant material

The diploid potato clones 87.1029-31, 87.1030-09 and 87.1031-29 are *amylose-free* as the result of a recessive mutation in the *GBSS* gene (*amf/amf*) responsible for amylose production in starch containing organs like tubers (Jacobsen *et al.*, 1989). These potato clones were used for *Agrobacterium* mediated transformation (Visser *et al.*, 1989) with the vector pMK1GBSS*Ac* (*Ac* T-DNA, Fig. 1; Pereira *et al.*, 1991) using the strain GV3101 (pMP90RK; Koncz and Schell, 1986). Primary transformants were indicated TM15-x, TM16-x and TM17-x where x stands for the independent transformant number.

For studying germinal transmission of autonomous *Ac* transposition a few selected TM17 primary transformants were crossed with diploid untransformed potato clones J91-6164-11 (El-Kharbotly *et al.*, 1994), J91-6167-2 (El-Kharbotly *et al.*, 1995) or J92-6570-6, derived from the cross J91-6164-1 (El-Kharbotly *et al.*, 1994) X J91-6146-15 (El-Kharbotly *et al.*, 1995). These *AmfAmf* potato clones were selected for *Phytophthora infestans R1* resistance.

Ds excision frequencies induced by the autonomous *Ac* element were studied after crossing primary *Ac* T-DNA transformants with *Ds* transposon containing (pHPT::*Ds*-Kan, Fig. 1; Pereira *et al.*, 1992) diploid potato clones BET92-Ds-A16-259 (Ds259), Ds53-22 and Ds53-34 (Ds53-22 and 34 are selected from the cross BET92-Ds-A16-Ds416 X J89-5040-20) (El-Kharbotly *et al.*, 1996a).

In vitro and in vivo analysis of Ac T-DNA transformants

The primary *Ac* T-DNA transformants were transferred to the greenhouse and assayed for ploidy level by counting the number of chloroplasts in stomata guard cells (Frandsen, 1968). All primary *Ac* T-DNA transformants were studied for somatic excision of *Ac* by monitoring the expression of the *GBSS* gene excision marker in tubers using iodine staining of starch (Kuipers *et al.*, 1994; Pereira *et al.*, 1991). For this purpose microtubers were induced on *in vitro* grown stem segments by placing them on MS medium (Murashige and Skoog, 1962) supplemented with 60 g l^{-1} sucrose

and 2 mg l^{-1} BAP. In the dark, at room temperature, microtubers were formed in the axillary buds after three to four weeks. *In vitro* grown microtubers and also mature greenhouse-grown tubers were analysed for starch composition by staining the surface of tuber slices with iodine (Kuipers *et al.*, 1994). In addition, starch granules were washed from the iodine stained tuber slices and the percentage of blue, red and blue-red staining starch granules was determined microscopically.

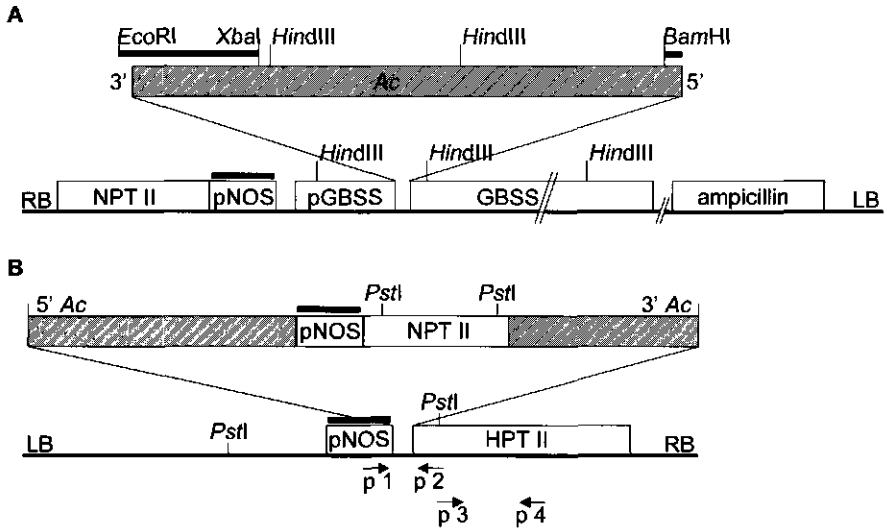

Fig. 1 Schematic drawing of (**A**) pMK1GBSS*Ac* and (**B**) pHPT::*Ds*-Kan showing the important restriction sites (vertical lines), probe positions (horizontal black lines) and positions of primer 1 (p1, gcg cgt tca aaa gtc gcc ta), primer 2 (p2, gtc aag cac ttc cgg aat cg), primer 3 (p3, aaa agt tcg aca gcg tct ccg acc) and primer 4 (p4, tct aca cag cca tcg gtc cag acg). Abbreviations: *LB* = left border, *RB* = right border, p*NOS* = nopaline synthase promoter, *NPT II* = neomycin phosphotransferase gene, p*GBSS* = granule bound starch synthase promoter, *GBSS* = granule bound starch synthase gene, *HPT II* = hygromycin phosphotransferase gene

Molecular analysis

Plant genomic DNA was isolated from leaves of greenhouse-grown plants (Pereira and Aarts, 1998) and used for Southern blot analysis to study the copy number of the *Ac* T-DNA integrations, the presence of the *Ds* T-DNA and the excision and re-insertion of

the *Ac* and *Ds* elements. The number of *Ac* T-DNA integrations in each transformant was determined by Southern blotting using *Hin*dIII digested genomic DNA probed with kanamycin (NPT II) and ampicillin (Amp) resistance gene fragments. The NPTII probe determines the right T-DNA border (RB probe) and the Amp probe the left T-DNA border (LB probe; Fig. 1). To study *Ac* excision and re-insertion, *Hin*dIII digested DNA was probed with either the complete *Ac* sequence (4.5 kb), the 5' site of *Ac* (0 - *Bam*HI site, 181 bp) or the 3' site of *Ac* (*Xba*I - *Eco*R1, isolated from a *wx-m7* clone; Behrens *et al.*, 1984; Fig 1). Expected *Hin*dIII fragments (Fig. 1) are the internal *Ac* *Hin*dIII restriction fragment (1605 bp), the 5' *Ac* up to the *Hin*dIII site in the *GBSS* gene (1935 bp) and the 3' *Ac* up to the *Hin*dIII site in the promoter region of the *GBSS* gene (2235 bp).

The presence of pHPT::*Ds*-Kan in seedlings was studied using *HPT* specific primers (p3 and p4, Fig. 1) in PCR. To reveal excision of the *Ds* transposon from the pHPT::*Ds*-Kan T-DNA, genomic DNA was restricted with *Pst*I and, after blotting, probed with the NOS promoter fragment (Fig. 1). With the NOS-promoter probe the *Ds* T-DNA revealed a hybridising *Pst*I fragment of 4.0 kb, the full donor site (FDS). After excision of *Ds* an empty donor site (EDS) of 2.3 kb is detectable. In plants that contain an *Ac* T-DNA, this analysis also displays a hybridising fragment with a size dependent on the site of integration of the *Ac* T-DNA. In this way the presence of *Ds* as well as the *Ac* T-DNA and excision of *Ds* could be studied in a single hybridisation analysis. A more rapid screening for *Ds* excision was performed using primers flanking the *Ds* excision site (p1 and p2, Fig. 1) resulting in an EDS-PCR fragment after excision of *Ds* (Enckevort *et al.*, 2000a; Chapter 3).

Results

Somatic Ac transposition in amylose-free (amf) potato background

1) Phenotypic analysis

Excision of the autonomous *Ac* element from the *Ac* T-DNA construct in tubers of an *amf* potato was previously described (Pereira *et al.*, 1991). Here, 40 of such *Ac* T-DNA transformants were studied in more detail with respect to their ploidy level, T-DNA

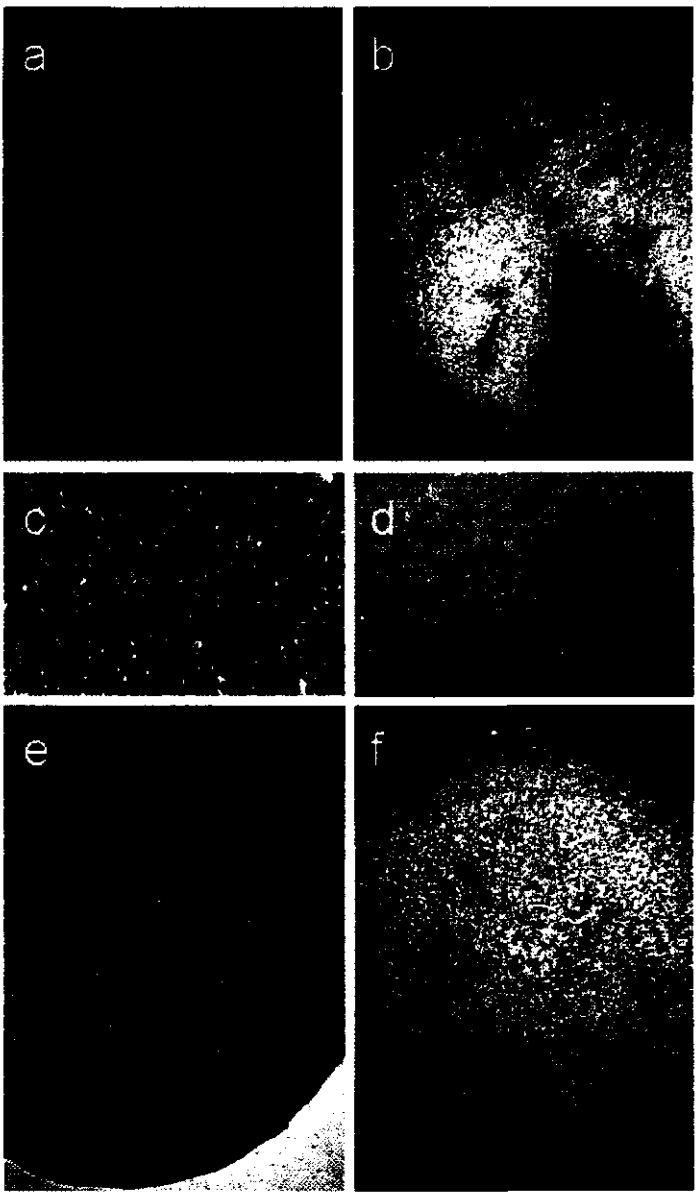

Fig. 2 *GBSS* gene expression observed on *amf*-mutant potato tuber slices after excision of *Ac* from the *GBSS* T-DNA transgene resulting in complementation of the *amf*-mutant. Blue-black coloured parts represent amylose staining and red or colourless parts represent the *amf*-mutant phenotype. a) complete reversion, class A; b) many small and larger reversion sectors, class B; c) class A detail with small red sectors probably due to re-insertion of the excised *Ac* in the *GBSS* transgene; d) class B detail including single cell excision; e) few reversion sectors, class C; f) no visible reversion, class D

copy number and *Ac* excision behaviour. By iodine staining of tuber slices and of individual starch granules *Ac* excision was monitored in the *amf* background. As expected, tuber slices of untransformed *amf* genotypes stained red with iodine. On tuber slices of the *Ac* T-DNA transformants different reversion patterns resulting from blue staining sectors became visible due to excision of *Ac* and expression of the *GBSS* transgene (Fig. 2). Starch granules were washed from these coloured tuber slices and the percentage of revertant starch granules was determined (Table 1).

Combining the data of tuber slice patterns and the frequency of revertant starch granules enabled a compilation of the 40 transformants into 4 classes (Fig. 2 and Table 1). Class A transformants (12 transformants) showed almost 100% revertant tuber slices and 96-100% revertant starch granules. Class B transformants (9 transformants) displayed various small and larger sectors of reversion on all tubers analysed and maximal 25% revertant starch granules. The small sectors of reversion on the tubers were sometimes even restricted to single cells. Transformant TM15-12 was classified intermediate between class A and B because some *in vitro* tubers showed complete reversion patterns (class A) while other tubers showed sectors with reversion (class B). Class C transformants (12 transformants) showed reversion sectors in only a part of the analysed tubers of each plant and 1-6% revertant starch granules. Class D transformants (6 transformants) did not show any visible reversion sectors or revertant starch granules.

Individual starch granules of the transformants showed three different colour phenotypes: red like in the *amf*-mutant indicating no reversion, normal blue indicating complete reversion, and blue-red indicating an intermediate phenotype. Table 2 shows a more detailed overview for the phenotype of the transformants that showed complete reversion (class A). Only one transformant, TM15-19, displayed an almost normal wildtype blue starch granule phenotype in *in vitro* tubers (97.2%) as well as in *in vivo* tubers (99.7%) indicating nearly complete *GBSS* gene complementation. Transformant TM15-28 displayed *in vitro* tubers with 59.5% and *in vivo* tubers with 56.4% blue staining starch granules. All other transformants predominantly showed intermediate blue-red staining starch granules. Transformants that displayed only sectors (1-25%) of reversion also had high percentages of blue-red instead of blue staining starch granules (data not shown). This intermediate *GBSS* complementation phenotype was found in 33% of the *amf*-mutants complemented with the *GBSS* genomic sequence (Flipse *et al.*, 1994).

Table 1 Analysis for ploidy level, T-DNA copy number and *Ac* excision from *Ac* T-DNA transformed diploid *amf*-mutants

Class[1]	Transformant	Genotype	Ploidy level	T-DNA copy	*in vitro* tubers		*in vivo* tubers	
					#[2]	%[3] blue and blue-red	#	% blue and blue-red
A	TM15-3	87.1029-31	2x	2	5	99	5	100
A	TM15-7	87.1029-31	2x	2	5	100	3	100
A	TM15-8	87.1029-31	2x	2	10	100	10	100
A	TM15-11	87.1029-31	2x	n.c.[4]	10	96	10	99
A	TM15-19	87.1029-31	2x	1	4	100	5	100
A	TM15-22	87.1029-31	2x	2	15	100	5	100
A	TM15-28	87.1029-31	2x	3	15	100	5	100
A	TM15-31	87.1029-31	2x	2	6	100	3	100
A	TM15-32	87.1029-31	2x	2	0	-	10	100
A	TM16-3	87.1030-09	2x	3	6	99	3	100
A	TM17-2	87.1031-29	2x	2	7	100	3	100
A	TM17-5	87.1031-29	2x	2	4	100	3	100
A/B	TM15-12	87.1029-31	4x	1	15	90	8	99
B	TM15-9	87.1029-31	2x	5	15	18	7	4
B	TM15-10	87.1029-31	2x	2	5	5	5	2
B	TM15-23	87.1029-31	2x	1	5	7	3	5
B	TM15-24	87.1029-31	2x	2	15	15	15	23
B	TM15-26	87.1029-31	2x	3	8	5	3	7
B	TM15-27	87.1029-31	2x	1	4	12	3	0
B	TM15-29	87.1029-31	4x	2	5	15	5	4
B	TM17-4	87.1031-29	2x	2	4	9	3	5
B	TM17-7	87.1031-29	2x	1	15	25	5	5
C	TM15-13	87.1029-31	2x	1	6	5	5	0
C	TM15-14	87.1029-31	-	2	4	4	5	5
C	TM15-15	87.1029-31	2x	1	15	4	5	1
C	TM15-17	87.1029-31	4x	0	5	1	3	0
C	TM15-18	87.1029-31	4x	1	4	2	3	1
C	TM15-25	87.1029-31	4x	2	4	1	3	0
C	TM16-1	87.1030-09	-	n.d.[5]	4	2	3	0
C	TM16-5	87.1030-09	4x	2	8	3	3	0
C	TM16-6	87.1030-09	2x	1	15	6	3	1
C	TM17-6	87.1031-29	4x	n.d.	0	-	3	1
C	TM17-8	87.1031-29	4x	2	7	5	3	1
C	TM17-9	87.1031-29	4x	n.d.	4	2	3	0
D	TM15-4	87.1029-31	4x	0	5	0	3	0
D	TM15-6	87.1029-31	4x	n.c.	6	0	3	0
D	TM15-30	87.1029-31	4x	n.c.	15	0	3	0
D	TM16-4	87.1030-09	4x	2	5	0	3	0
D	TM16-7	87.1030-09	2x	n.c.	6	0	3	0
D	TM17-10	87.1031-29	2x	1	8	0	3	0
	control	87.1029-31	2x	0	5	0	5	0
	control	87.1030-09	2x	0	5	0	5	0
	control	87.1031-29	2x	0	5	0	5	0

[1] Classification by pattern as displayed in Fig. 2, [2] Number of analysed tubers, [3] % revertant starch granules (see text), [4] n.c. = no complete T-DNA detected, [5] n.d. = not determined

In three of the transformants of class A (TM15-08, TM15-11 and TM15-32) some tuber slices displayed small sectors without *GBSS* gene expression (Fig. 2). These are probably due to re-insertion of the excised *Ac* in the *GBSS* transgene. Almost all transformants of class A and B appeared to be diploid, while tetraploid transformants predominantly exhibited small or no reversion sectors and were in class C or D. No clear differences were observed between axenically and soil grown tubers.

Table 2 The number of blue, blue-red and red staining starch granules and the frequency of blue granules from class A transformants

Transformant	*in vitro* tubers				*in vivo* tubers			
	blue	blue-red	red	% blue	blue	blue-red	red	% blue
TM15-03	27	2152	28	1.20	10	2213	3	0.45
TM15-07	40	2075	0	1.90	25	2648	1	0.94
TM15-08	49	2163	6	2.20	26	2056	2	2.20
TM15-11	87	2139	90	3.80	40	2063	15	1.90
TM15-19	2592	63	12	97.20	2333	7	1	99.70
TM15-22	1	2875	0	0.04	14	2990	8	0.46
TM15-28	2265	1536	8	59.50	3290	2534	14	56.40
TM15-31	5	2722	10	0.18	2	3100	6	0.06
TM15-32	-	-		-	72	2031	2	3.40
TM16-03	27	2152	28	1.20	1	3000	5	0.03
TM17-02	27	3540	0	0.76	2	3375	2	0.06
TM17-05	392	2760	8	12.40	6	2617	2	0.23
TM15-12	100	2490	275	3.00	95	2213	20	4.10
wildtype				100.00				100.00
amf-mutant				0.00				0.00

2) Molecular characterisation of *Ac* T-DNA's and *Ac* excision

The T-DNA copy number of all 40 *Ac* T-DNA transformants was determined by analysing *Hin*dIII digested genomic DNA hybridised to the RB and LB T-DNA probes (Table 1). Four transformants of class D showed no or an incomplete T-DNA insertion explaining the absence of *GBSS* gene reversion in these transformants. TM15-3 and TM15-7 showed identical T-DNA insertion fragments, which indicated that they are similar regenerants from one transformation event.

From the four reversion classes, a set of 15 mostly diploid *Ac* T-DNA transformants with 1 or 2 T-DNA copies was further analysed for *Ac* excision by DNA blot hybridisation using the complete *Ac* as probe (Fig. 3). From the six analysed plants of class A transformants with 100% reversion, five plants (lane 11-14, 16)

lacked the full donor site (FDS) which corresponded to the 5' and 3' ends of *Ac* in the T-DNA. Transformant TM17-5 (lane 15) with two *Ac* T-DNA loci showed one early excision and one remaining FDS. These molecular observations on leaf material confirmed the phenotypes of early *Ac* excision for which these plants were selected using the *GBSS* gene excision marker. All class A plants showed new *Ac* insertion sites visible as additional hybridising fragments. In TM15-11 (lane 11) two strong, one intermediate and two weak hybridising *Hin*dIII fragments are visible besides the internal *Ac Hin*dIII fragment c, indicating the presence of 2 or 3 new *Ac* insertions. After hybridisation of this same blot with the 5' *Ac* probe, revealing *Ac* left junction fragments (indicated with * in Fig. 3), it was concluded that at least 2 *Ac's* transposed early and the remaining weak hybridising fragment is very likely a third *Ac*. However, this third *Ac* was not identified with the 5' *Ac* probe indicating a deletion of this *Ac* that has resulted from a somatic transposition event. In analysing the *Ac* T-DNA copy number of TM15-11 (Table 1), the T-DNA seemed to be deleted or partly integrated. Still two or three *Ac* copies were present, probably due to multiplication of one original *Ac*. In TM15-8, a 2-copy T-DNA insertion was identified (Table 1) but only one *Ac* re-insertion (lane 12) is visible on the blot represented by three hybridising *Hin*dIII fragments from one single *Ac* (confirmed with 5' *Ac* probe). The two identical transformants, TM15-3 and TM15-7 (lane 13 and 14), showed identical re-insertion sites for the two *Ac*'s present. This indicates early *Ac* transposition in the primary transformed tissue and low somatic transposition after regeneration. The 2-copy number transformant TM17-5 (lane 15) showed both an original *Ac* T-DNA (fragments a, b and c) as well as a new *Ac* insertion site. In TM17-2 (lane 16) the two original *Ac* T-DNA integration sites were completely empty (absence of fragments a and b) and both *Ac* elements re-inserted at new positions.

Molecular analysis of four of the nine transformants from class B showed the presence of the original *Ac* T-DNA configuration in all cases (Fig. 3, lanes 7 to 10, fragments a, b and c). The phenotypically visible reversion in the tuber starch granules, was not revealed in *Ac* re-insertions except for TM17-4 (lane 10). This transformant with two T-DNA copies showed a unique hybridisation pattern with 6 complete *Ac* hybridisation fragments corresponding to 3 new *Ac* re-insertion sites (confirmed with 5' *Ac* probe). This is similar to TM15-11, an example of multiplication of *Ac* transposons.

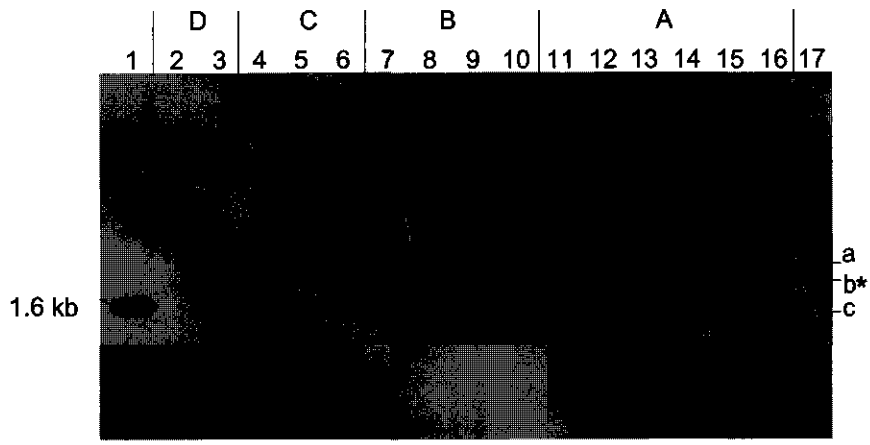

Fig. 3 *Hind*III digested genomic DNA hybridised to a complete *Ac* probe. Fragment a and b correspond to the full donor site (FDS) *Hind*III fragments of the 3' site (2235-bp) and the 5' site (1935-bp) of *Ac* in the *Ac* T-DNA. Fragment c is the *Ac* internal *Hind*III fragment present in *Ac* transformants (1605-bp). Fragments marked with * are new *Ac* insertions that displayed hybridisation with the 5' *Ac* probe. *Lane 1* shows the 1.6 kb marker hybridisation; *lane 2* and *3* are class D plants TM15-4 and TM17-10; *lane 4* to *6* are class C plants TM16-6, TM15-15 and TM15-13; *lane 7* to *10* are class B plants TM17-7, TM15-24, TM15-23 and TM17-4; *lane 11* to *16* are class A plants TM15-11, TM15-8, TM15-3, TM15-7, TM17-5 and TM17-2; *lane 17* is an untransformed control

From class C, phenotypically displaying 1- 6% reversion in starch granules, three plants were analysed molecularly and two of them did not show re-insertion of an *Ac* element as could be expected from the late and low level of reversion in tubers (Fig. 3, lanes 4 to 6). However, TM15-15 (lane 5) exhibited a new *Ac* hybridising fragment and a strong hybridisation of the 1935-bp 5' *Ac* T-DNA fragment. This indicates one *Ac* excision and re-insertion despite the detected very low levels (4 - 1%) of reversion with amylose synthesis in the analysed tubers. As TM15-15 contained only one complete T-DNA, judged from the RB and LB probe hybridisations (Table 1), the transposed *Ac* may be from an incomplete or lost T-DNA insertion.

From class D without reversion in tuber slices, two transformants (Fig. 3, lane 2 and 3) were analysed. TM15-4 (lane 2) showed no hybridisation and was thus not transformed. Surprisingly, TM17-10 (lane 3) revealed excision and transposition. One complete *Ac* T-DNA seemed to have given rise to a new *Ac* insertion and two

additional deleted *Ac*'s. This is revealed by three 5' *Ac* hybridising fragments (*) and a remaining 3' *Ac* fragment. In this transformant a deletion or mutation of the transgenic *GBSS* gene resulting from *Ac* transposition could probably explain the difference in *GBSS* complementation in somatic tuber tissue and the transposition observed at the DNA level.

Table 3 Summarised data for the three selected TM17 transformants used for crossings

	TM17-2	TM17-4	TM17-5
Reversion class	A	B	A
Right border NPT II probe	1	nd	nd
Left border ampicillin probe	2	1	2
Copy number	2	2	2
Complete *Ac* T-DNA	-	1	1
Ac re-insertion	1½	3	1

nd = not determined

3) Description of selected *Ac* crossing parents

Class A transformants TM17-2 and TM17-5 and the class B transformant TM17-4 were used as parents in crosses to study germinal transmission of *Ac* transposition to next generations and to study induction of *Ds* transposition by combining *Ac* and *Ds* in these plants. Table 3 summarises the data obtained for these three primary transformants. The primary class A transformant TM17-2 (Fig. 3, lane 16) contains 2 T-DNA's in inverted orientation as determined with RB and LB T-DNA probes. Complete early *Ac* excision of the 2 *Ac*'s was detected on analysing the *Hin*dIII fragments probed with the complete *Ac* (Fig. 3). The largest fragment corresponds to the 5' site of a new insertion site of *Ac* (confirmed by hybridisation with the 5' *Ac* probe). Fragment c of about 1.6-kb corresponds to the internal *Hin*dIII fragment of *Ac*. The two lower fragments are almost equal in size and correspond to two 3' sites of *Ac* insertions. From these observations we conclude that two *Ac* T-DNA's were present in the primary transformant, both *Ac*'s excised with one deleted at the 5' site (d*Ac*) and the other being a complete functional *Ac*.

The primary class B transformant TM17-4 represents a 2-copy number plant with high levels of *Ac* transposition. In leaf material this transformant exhibited one original T-DNA insertion together with three *Ac* re-insertions at new positions (Fig. 3,

lane 10). The three smallest fragments (fragments a, b and c) corresponded to the original *Ac* T-DNA configuration (1605, 1935 and 2235-bp). From the new fragments the three strong hybridising fragments correspond to the 5' site of new *Ac* insertions (confirmed by hybridisation with the 5' *Ac* probe). From these observations it is concluded that TM17-4 contained two T-DNA copies in inverted orientation including probably one incomplete copy. The presence of both an original *Ac* T-DNA and transposed *Ac*'s suggests that at least one *Ac* copy transposed early in development and amplified to multiple insertion sites. This same phenomena of rapid multiplication of a single *Ac* was earlier found in tomato (Yoder, 1990).

The primary class A transformant TM17-5 (Fig. 3, lane 15) contained one original *Ac* T-DNA and one new *Ac* insertion visible as two additional fragments, one corresponding to the 3' site and the other one to the 5' site of *Ac* (confirmed by hybridisation with the 5' *Ac* probe).

Parents	Progeny genotype						
Ac ♀ X - ♂	*Ac* present						*Ac* absent
	Parental *Ac*'s linked				Parental *Ac*'s unlinked		
		Parental *Ac*'s	With new somatic *Ac*	New *Ac* position	*Ac* T-DNA	*Ac* re-insertion	
TM17-2 X J91-6164-11	13	7	4	2			9
TM17-4 X J91-6167-2					1 (1 *Ac* T-DNA and 1 *Ac* re-insertion)		
TM17-5 X J92-6570-6	4	2	1	1	5	1	7

Fig. 4 Scheme showing *Ac* segregation analyses in sexual progeny

Germinal transmission of autonomous Ac transposition events

Transformant TM17-2 of class A was crossed with J91-6164-11 and 22 seedlings were analysed for the segregation of *Ac* excision and for new *Ac* excision and re-insertions. Fig. 4 shows the *Ac* segregation for all progeny seedlings determined by DNA blot analyses of *Hin*dIII digested genomic DNA probed with the 3' *Ac* fragment (Fig. 5). Thirteen seedlings (7+4+2) inherited two *Ac* loci (of which one is the incomplete *Ac*, d*Ac*) while nine did not inherit an *Ac*. This clearly indicates monogenic inheritance and

linkage between the two *Ac* loci in the original TM17-2 transformant ($\chi^2_{1:1}$ = 0.72, p > 0.1). Two of the thirteen *Ac* containing seedlings showed independent new *Ac* insertion sites due to transposition of the functional *Ac* element (Fig. 5, lane 2 and 3). In these seedlings *Ac* excision events were very likely present in the gamete and transmitted to the progeny (germinal excisions). Additional somatic *Ac* transposition events (lane 1) were observed in 4 of the other eleven *Ac* containing seedlings.

Seeds from the cross TM17-4 (class B) X J91-6167-2 germinated very poorly and only one seedling could be analysed (Fig. 4). It contained one of the three *Ac* re-insertions together with the original *Ac* T-DNA insert, indicating that probably not all *Ac* re-insertions were closely linked in the original transformant TM17-4 leading to segregation of the *Ac* elements in the gametes. No new *Ac* re-insertions were found in this seedling.

TM17-5 (class A) was crossed with J92-6570-6 and 17 seedlings were analysed (Fig. 4). Four seedlings inherited both the *Ac* T-DNA as well as the *Ac* re-insertion, 5 seedlings inherited only the *Ac* T-DNA, one seedling inherited only the *Ac* re-insertion and 7 seedlings inherited no *Ac*. Since all four segregation classes were found in these 17 seedlings, the two *Ac* positions seemed to segregate independently. From the four seedlings with both the *Ac* T-DNA as well as the *Ac* re-insertion, in one seedling the *Ac* re-insertion is at a new position indicating germinal transmission of this late *Ac* transposition. Another seedling of these four contained additional new somatic *Ac* insertions.

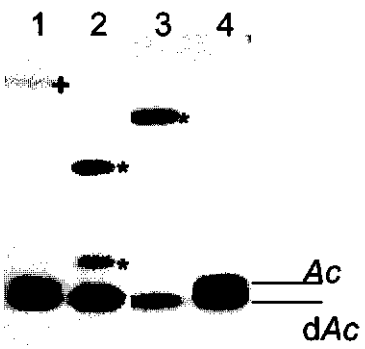

Fig. 5 *Hin*dIII digested genomic DNA hybridised to the 3' *Ac* probe. *Lane 4* shows the *Ac* and d*Ac* fragments present in the *Ac* parental line TM17-2. *Lane 1* to *3* represent 3 progeny plants from the coss TM17-2 X J91-6164-11. In both *lane 2* and *3* new positions of the active *Ac* are present (*). In *lane 1* a somatic *Ac* is present (+)

Somatic Ds transposition due to presence of an autonomous Ac

Ds excision induced by introduction of a functional *Ac* element via crossing was earlier investigated in the progeny of crosses between TM17-2 (class A) and Ds53-22 or Ds53-34. Two populations of respectively 18 and 96 seedlings were analysed both molecularly and phenotypically for presence of *Ds* T-DNA, *P. infestans R1* resistance and *Ac* induced *Ds* transposition (Enckevort *et al.*, 2000a; Chapter 3). From the segregation data (Table 4) it was concluded that plants containing both *Ac* and *Ds* always showed active somatic *Ds* excision detected by PCR. The *Ds* excision seemed to occur relatively late in development resulting in chimaeric plants with small sectors (about 10 to 15% of the cells) of independent excision events (Enckevort *et al.*, 2000a; Chapter 3). Only one progeny seedling in these studies showed complete *Ds* excision early in development. This is a rather low frequency (5%) compared to the number of early *Ac* excisions in the *Ac* primary transformants (32.5%).

Table 4 *Ds* segregation analyses and identified *Ds* transposition indicating presence of *Ac* after crossing of several different *Ac* and *Ds* lines

Parents	Progeny				
Ds ♀ X *Ac* ♂	Total	*Ds* present			
			transposed *Ds*	stable *Ds*	nd
Ds53-22 X TM17-2	18	8	3	5	
Ds53-34 X TM17-2	96	39	19	20	
Ds53-34 X TM17-4	77	33	32	0	1
Ds259 X TM17-4	5	3	3	0	

nd = not determined

To substantiate the earlier reported results here, from a cross between Ds53-34 and TM17-4 (class B), 77 seedlings were analysed by PCR for presence of the *Ds* T-DNA construct (Table 4). As expected, the *Ds* T-DNA insert segregated in a 1:1 ratio in this population ($\chi^2_{1:1} = 0.05$, $p > 0.5$). From the 33 plants that possessed the *Ds* T-DNA, 32 plants were analysed by EDS-PCR and 22 also by DNA blot analyses. All the 32 plants analysed showed active *Ds* excision (data not shown) indicating that they contained an active *Ac* element. Compared to seedlings with TM17-2 as parent, the excision percentage in these plants was higher, estimated by band intensity and varied between 10 and 25%. This observation suggests that most of the TM17-4 progeny

plants contained more than 1 *Ac* transposon copy that confers a higher level of somatic *Ds* excision compared to the progeny of TM17-2 which contained only one active *Ac* element.

TM17-4 (class B) was also crossed with Ds259, a second *Ds* T-DNA transformant. Five seedlings were obtained and four of them were analysed by PCR and DNA blot analyses. Three plants contained the *Ds* T-DNA and at least one *Ac* element. *Ds* excision was shown in all cases by the presence of an EDS fragment. Thus also this *Ds* T-DNA (Ds259) locus showed *Ds* excision in the presence of an active *Ac* transposase source.

Discussion

Autonomous Ac transposition in primary diploid potato transformants

High levels of *Ac* excision in primary *Ac* T-DNA transformants of diploid potato clones was visually detected using the *GBSS* marker gene. In 32.5% (13 out of 40) of the primary transformants, early *Ac* excision immediately after transformation resulted in a complete *GBSS* revertant allele. Molecular analyses of leaf DNA confirmed early *Ac* excision (class A) by showing empty donor sites in all tissues tested and new *Ac* re-insertion sites. This high number of transformants with complete *Ac* excision indicated that directly after transformation the expression of the *Ac* transposase gene was sufficient to induce *Ac* excision and subsequent re-insertion in the primary transformed cells. In most of these transformants only a few *Ac* transpositions are visible by DNA blot hybridisation suggesting a limited number of cells involved in the primary transposition event. Most probably transposition occurred in the first transformed cell, as the *GBSS* revertant phenotype is observed homogeneously throughout the plant. Such high frequency of autonomous *Ac* transposition directly after transformation has not yet been documented before. Probably in these transformants the *Ac* T-DNA has inserted in positions of the genome that induce early transpositions. Since these are one third of the *Ac* T-DNA positions (32.5%) they might be near transpositionally active regions, in or near highly expressed genes. The number of *Ac* T-DNA copies seems to be less important since different copy numbers were represented in this high frequency reversion class A.

Three transformants (TM15-08, TM15-11 and TM15-32) displayed very small mutant sectors lacking *GBSS* gene expression in an almost complete revertant background (Fig. 2). Probably these are either sectors of re-insertion of the excised *Ac* back into the *GBSS* transgene or *GBSS* gene silencing. This can be due to efficient *Ac* re-insertion at linked sites in the chromosome (Hehl and Baker, 1990).

The majority (60%) of the primary *Ac* T-DNA transformants displayed variegated patterns (class B and C) of *Ac* excision that demonstrated the utility of the *GBSS* marker gene to exhibit somatic excision in potato tubers. The occurrence of many small and some large sectors of *GBSS* gene reversion indicated continuous independent *Ac* transposition at different developmental stages and at different time points. Also many single cells diplaying *Ac* excision were observed (Fig. 2d). Since these independent *Ac* re-insertions are only present in a few cells they are not detectable by DNA blot analyses. This makes the *GBSS* gene in potato, like was found for the other cell autonomous genes in other species (Finnegan *et al.*, 1989; Goldsbrough *et al.*, 1996; Jones *et al.*, 1989; Spena *et al.*, 1989), a more sensitive measure of *Ac* excision than DNA blot analysis. Simultaneously, rapid proliferation of *Ac* copies was detected in one of the transformants supporting the model that amplification of *Ac* is associated with transposition (Yoder, 1990). In this way a rapid increase in the copy number of *Ac* transposable elements can occur in potato like in other heterologous plants.

Among single copy plants, all patterns of variegation, from class A to D were present suggesting that it is the position of the *Ac* T-DNA in the genome that contributes most to the excision phenotype. The T-DNA copy number in class B and C transformants, varying from 1 to 5 copies, has presumably less influence on the *Ac* transposition behaviour. These observations underpin the earlier suggestion that *Ac* T-DNA positions in the potato genome determine the transposition phenotype (Pereira *et al.*, 1991) and strongly supports the position hypothesis proposed by Peterson (1977).

One remarkable observation is the low frequency of excisions (class C and D) observed in tetraploid plants (Table 1). This could be due to silencing of *GBSS* gene expression or to low levels of transposase activity in tetraploid transformants. This confirms the earlier discussed observation on the surprisingly high levels of early transposition among diploid potato transformants.

GBSS gene reversion

The cell autonomous expression of the *GBSS* gene was shown to be an accurate visual *Ac* excision marker for *in vitro* microtubers and greenhouse-grown mature tubers. Due to the possibility to detect *GBSS* gene expression at very low levels, in 85% (34 out of 40) of the primary *Ac* T-DNA transformants blue staining starch was found revealing reversion leading to complementation. This percentage is similar to *GBSS* gene complementation obtained after transformation of the *amf* mutant with the *GBSS* genomic sequence driven by its own promoter (Flipse *et al.*, 1994). This similarity in complementation frequencies confirms the occurrence of autonomous *Ac* excision in almost all potato transformants.

More specific analyses on the reversion phenotype of starch granules in amyloplasts showed that the majority did not display the exact wildtype phenotype but an intermediate phenotype with blue-red staining starch granules (94% of the complemented transformants). In the complementation studies with a genomic *GBSS* gene this intermediate phenotype was found in only 33% of the transformants (Flipse *et al.*, 1994). The site of T-DNA integration and silencing due to multiple integrations are possible explanations for the intermediate phenotypes with low or unstable *GBSS* gene expressions. The T-DNA with *GBSS Ac* accommodated in addition to the *Ac* element a 60-bp sequence from the waxy (*wx-m7*) locus of maize (Behrens *et al.*, 1984). After *Ac* excision this 60-bp fragment remains present between the transcription and translation start of the *GBSS* transgene. As described previously after excision of an *Ac* or *Ds* element the remaining waxy gene sequences in the untranslated leader sequence of *rolC* (Spena *et al.*, 1989) or *Lc* (Goldsbrough *et al.*, 1996) influenced the expression level of these genes, which may also be the case for the *GBSS* gene. It is known that the presence of a 140-bp sequence in *GBSS* gene promoter region reduces expression, and deleting this 140-bp sequence improves the *GBSS* gene activity (Wal *et al.*, 2000). This suggests that the *GBSS* gene is suitable for semi-quantitative gene expression analyses like shown in antisense and sense inhibition studies (Kuipers *et al.*, 1994). In our study only one transformant showed complete reversion to wild type phenotype and in one transformant about 50% of the granules were reverted to wild type. In these blue starch granules *GBSS* gene expression is fully complemented to the wildtype expression level. In this specific case the *GBSS* gene expression is either influenced by a larger deletion, including the additional 60-bp

waxy sequence due to *Ac* excision or by a position effect on the T-DNA integration site.

Germinal transmission of autonomous Ac transposition events

In sexual progeny of the transformants TM17-2 (class A), TM17-4 (class B) and TM17-5 (class A) respectively 13, 1 and 10 *Ac* containing seedlings were analysed for *Ac* positions. In most of these progeny seedlings the *Ac* positions were identical to the parental transformant. In 3 seedlings a new *Ac* insertion position, not identified in the parental transformant,was determined. The occurrence of these new *Ac* insertions in progeny and the absence of the parental *Ac* positions, indicates germinal transmission of somatic *Ac* transpositions. This shows that production of *Ac* insertion lines in potato through seeds is a potential method for the developing of transposon insertion mutants.

TM17-2 and TM17-5 were selected for their early *Ac* excision. *Ac* T-DNA was probably inserted in a region conferring early or active transposition. In their progeny seedlings the transposed *Ac* re-insertions were less active showing later *Ac* excision than in the primary transformant. Similar to that shown in tobacco (Taylor *et al.*, 1989), *Ac* excision pattern in the primary potato transformants is thus not a reliable indicator for *Ac* activity in the progeny. However, somatic *Ac* excision events still occurred in the progeny seedlings indicating that the expression of *Ac* continues, but at a different level in these seedlings when compared to the primary transformant.

Somatic Ds transposition due to presence of an autonomous Ac

After crossing the *Ac* containing transformants with *Ds* containing potato clones, somatic *Ds* excision was found to occur in all seedlings that inherited both *Ds* and *Ac*. EDS-PCR and DNA blot analyses confirmed somatic *Ds* excision levels varying from 10% in TM17-2 progeny seedlings (Enckevort *et al.*, 2000a; Chapter 3) to 25% in TM17-4 progeny seedlings containing the amplified *Ac* elements. In only one TM17-2 progeny seedling early *Ds* excision was detected. This single early *Ds* excision among 57 analysed *Ac-Ds* plants is a low number in contrast to the primary transformants for

which in at least 32.5% of the transformants early *Ac* excision in the first cells was observed. This could imply a difference between autonomous *Ac* transposition and *in trans Ds* transposition frequencies. However, the activity of the *Ac* transposase promoter could be different in the original locus compared to the new insertion locus in the genome. *Ac* transposition from the original T-DNA is often early, while in progeny *Ac* excision is frequently late and thus similar to the excision pattern induced on the *Ds* elements.

The developed *Ac-Ds* seedlings are highly chimaeric plants with high levels of late independent *Ds* insertions that are suitable to produce a tagging population of independent transpositions (Enckevort *et al.*, 2000a; Chapter 3). The resulting population is directly useful for the isolation of specific target genes that are heterozygous. The observation that the rate of somatic excision of an *Ac* or *Ds* element is low (to maximal 25%), will result in mutants that will be phenotypically mutant in the majority of tissue and thus easily distinguishable.

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Chapter 3

Selection of independent *Ds* transposon insertions in somatic tissue of potato by protoplast regeneration

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Abstract

Potato is an autotetraploid crop plant that is not very amenable to the deployment of transposon tagging for gene cloning and gene identification. After diploidisation it is possible to get potato genotypes that grow well, but they are self-incompatible. This prevents the production of selfed progeny that are normally used in gene tagging approaches to select for parental lines with the target gene to be tagged in a homozygous stage. We describe here an alternative selection method for directed transposon tagging for a gene of interest in a heterozygous background. Diploid potato plants with a *Ds* transposon linked to the desired gene of interest (the *Phytophthora infestans R1* resistance locus) in a heterozygous stage were used for the development of this directed transposon tagging strategy. After crossing to a diploid *Ac* transposon-containing genotype, 22 'interesting' seedlings (R1Ds/r-;Ac/-) were selected that showed active *Ds* transposition as displayed by DNA blot hybridisation, empty donor site PCR and sequencing. Protoplast isolation and the use of the hygromycin gene as a cell-specific selection marker of *Ds* excision enabled the direct selection of *Ds* excision sectors in these highly chimaeric seedlings. This somatic selection of *Ds* transpositions and the regeneration through protoplasts resulted in the development of a large population of almost 2000 hygromycin-resistant plants. Southern blot analysis confirmed the insertion of *Ds* at independent positions in the genome. Every selected plant displayed independent *Ds* excisions and re-insertions due to the expression of the *Ac* transposase throughout development. This population, which is developed from seedlings with the desired *R1* gene in a heterozygous stage is directly useful for searching for transposon-tagged *R1* mutants. In general, this approach for selecting for somatic transpositions is particularly suitable for the molecular isolation of genes in a heterozygous crop like potato.

Introduction

McClintock's genetic studies in maize resulted in the characterisation of *Dissociation* (*Ds*) and *Activator* (*Ac*) elements that cause chromosome breakage and mutations (McClintock, 1948; 1950). The molecular isolation and characterisation of this two-component maize *Ac-Ds* transposable element (Fedoroff *et al.*, 1983) directly lead to the isolation of genes that were 'tagged' by these elements (Fedoroff *et al.*, 1984). *Ac-Ds* and other maize transposable elements (*En/Spm*) are also functional tools for isolating genes in several heterologous plant species like Arabidopsis (Aarts *et al.*, 1993), petunia (Chuck *et al.*, 1993), tobacco (Whitham *et al.*, 1994), tomato (Jones *et al.*, 1994) and flax (Lawrence *et al.*, 1995). These are all self-fertilising plant species. Either random tagging strategies (Arabidopsis, petunia) by screening large populations for mutants or the targeted tagging of specific genes (tobacco, tomato, flax) were applied. In addition, positive selection strategies (tobacco and tomato) facilitated the direct selection of a mutant phenotype. By self- or testcrossing, large populations were produced for the direct screening of possible transposon-tagged mutants. The number of the germinal transmissions of transposon insertions determines the number of useful mutation events in such large selfed progeny populations.

Phytophthora infestans causes potato late blight and is one of the most economically important diseases that occurs in the autotetraploid potato crop. A large number of *R* (resistance) genes are present in the hexaploid wild species *Solanum demissum* and its introgressions in tetraploid potato cultivars. These race-specific *R* genes do not provide durable field resistance because of the rapid evolution of new virulent races of the fungus. To improve understanding of the molecular basis of *R* gene-mediated disease resistance we initiated a transposon tagging strategy in diploidised potato aimed at the molecular isolation of the *S. demissum R1* gene. Knapp *et al.* (1988) and Pereira *et al.* (1991) had previously shown that the maize *Ac* transposable elements are functional in potato. To tag the *R1* gene, diploid potato genotypes were induced and, after crossing, selected for a high *Agrobacterium tumefaciens* transformation frequency and segregation for the *R1* gene (El-Kharbotly *et al.*, 1995), earlier mapped on chromosome 5 (Leonards-Schippers *et al.*, 1992). Self-incompatibility at the diploid level hindered the production of vigorous homozygous plants, and the selection of *R1* homozygous diploid potato turned out to be a difficult and time-consuming process (unpublished). Therefore, an alternative

method was developed to employ directed transposon tagging in diploid potato that was heterozygous for *R1*.

A common property of transposable elements is somatic instability within a plant, displayed as variegation or chimerism for wild-type and mutant sectors. Visual transposon phenotypic excision assays with marker genes encoding streptomycin resistance (Jones *et al.*, 1989), β-glucuronidase (*GUS*; Masson and Fedoroff, 1989), *rolC* (Spena *et al.*, 1989) or *GBSS* (Pereira *et al.*, 1991) made somatic excision events screenable. In this way the occurrence and frequency of transposition could be determined and used for the selection of excision events. When kanamycin (Baker *et al.*, 1987) or hygromycin (Rommens *et al.*, 1992a) marker genes are used, the selection of excision events at the cellular level is feasible and, in combination with effective *in vitro* selection and somatic propagation procedures, the production of large numbers of transposon mutants can be facilitated.

Diploid potato plants heterozygous for the *P. infestans R1* resistance gene were transformed with an *Agrobacterium* strain containing a *Ds*-transposon T-DNA construct. After mapping of the *Ds* T-DNA insertions (Jacobs *et al.*, 1995), two plants harbouring the *Ds* T-DNA on chromosome 5 were further propagated. Sexual progeny of one of these *Ds* plants was obtained, and plants with the *R1* gene and the *Ds* T-DNA in coupling phase (18 cM) were selected (El-Kharbotly *et al.*, 1996a). By further crossing, an active *Ac* was introduced for the induction of *Ds* transposition.

This paper describes the selection of *R1* resistant diploid potato plants with an *R1*-linked *Ds* showing transposition late in development producing a population of highly chimaeric plants. Instead of searching for germinal transmission, we selected directly for somatic excision events by protoplast isolation and the use of hygromycin as a cell-specific selection marker for *Ds* excision. In this way a population of hygromycin-resistant regenerants was produced, and Southern blot analysis confirmed the selection of independent *Ds* mutations. This approach shows that somatic selection for excision and probably independent transposition events can facilitate the production of large tagging populations. This is particularly suitable for the isolation of genes in heterozygous crops like potato.

Materials and methods

Plant material

The diploid potato clone J92-6400-A16, *R1* resistant against *P. infestans* (R1r1), was transformed with the *Ds* transposon-containing T-DNA construct pHPT::*Ds*-Kan shown in Fig. 1 (El-Kharbotly *et al.*, 1995; Pereira *et al.*, 1992). Transformants BET92-Ds-A16-259 (Ds259) and BET92-Ds-A16-416 (Ds416) were selected for a *Ds* T-DNA insertion on chromosome 5 (El-Kharbotly *et al.*, 1996a). Both were linked in repulsion phase to the previously mapped *P. infestans R1* resistance gene (Leonards-Schippers *et al.*, 1992). Crossing Ds259 to diploid genotypes susceptible for *P. infestans* resulted in very few progeny. Crossing Ds416 to the same susceptible diploid genotypes resulted in offspring and enabled the selection of four recombinant plants (Ds53-3, -15, -22 and -34) having the *R1* gene and the *Ds* T-DNA in coupling phase (18 cM) (El-Kharbotly *et al.*, 1996a). In Ds416 two pHPT::*Ds*-Kan copies were present at one locus (Jacobs *et al.*, 1995).

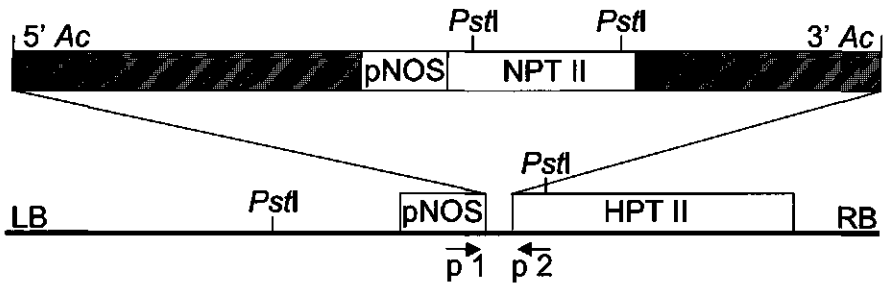

Fig. 1 Schematic drawing of pHPT::*Ds*-Kan showing the positions of primer 1 (p1, gcg cgt tca aaa gtc gcc ta), primer 2 (p2, gtc aag cac ttc cgg aat cg) and *Pst*I restriction sites. *LB* = left border, *RB* = right border, *pNOS* = nopaline synthase promoter, *NPT II* = neomycin phosphotransferase gene, *HPT II* = hygromycin phosphotransferase gene

Seeds were generally germinated on MS medium (Murashige and Skoog, 1962) supplemented with 30 mg l^{-1} sucrose and 8 g l^{-1} agar (MS30). Shoot tips or axillary buds from *in vitro*-grown seedlings were used for the *in vitro* selection of kanamycin- and hygromycin resistant seedlings. After transfer to the greenhouse all seedlings

were tested for *P. infestans R1* resistance as described previously (El-Kharbotly *et al.*, 1994), using *P. infestans* 89148-09 (race 0, kindly supplied by Dr. F. Govers, Laboratory of Phytopathology, Wageningen University and Research Centre, The Netherlands).

Molecular analysis

Plant genomic DNA was isolated from greenhouse-grown plants according to Pereira and Aarts (1998). Primers were designed on pHPT::*Ds*-Kan and the polymerase chain reaction (PCR) was used to show excision of *Ds* (Fig. 1). Southern analysis was performed using *Pst*I restricted genomic DNA and the blot probed with a NOS promoter DNA fragment. With this probe both the *Ac* as well as the *Ds* T-DNA loci revealed hybridising fragments. PCR products were separated on a 0.8% TBE agarose gel, and the specific empty donor site PCR fragments (EDS-PCR) were cut out of the gel. DNA was isolated from the agarose using Qiaex II (Qiagen), and the EDS-PCR fragments were cloned in a pGEM ¯T easy vector (Promega Corp) and sequenced using an automated ABI 373 DNA sequencer.

Protoplast isolation, culture and regeneration

Protoplasts were isolated from 4-week-old *in vitro*-grown shoots according to Uijtewaal *et al.* (1987). Before cutting, the plants were placed in the dark at 4°C for 6 h. After the second centrifugation step the protoplast pellet was not further purified but immediately re-suspended in culture medium TM2G (Wolters *et al.*, 1991) to a final concentration of 500,000 pp ml^{-1}. In the first week the protoplast cultures were diluted 1:1 several times with fresh TM2G medium, and in the second week they were diluted 1:1 with TMD medium (Wolters *et al.*, 1991). In the third week the calli were transferred to callus growth medium, and after 2 weeks of growth the largest calli were collected on shoot induction medium (Mattheij *et al.*, 1992). Finally the calli were maintained on shoot elongation medium (Mattheij *et al.*, 1992) until regenerated plants could be harvested. To select specifically for protoplast regenerants with excision events, we added 10 mg l^{-1} hygromycin to the callus growth medium 14 days after protoplast isolation. The

hygromycin concentration was increased to 20 mg l^{-1} on day 21 and maintained during the whole protocol. In additional selection experiments the hygromycin concentration in the culture media was increased to 30 mg l^{-1} and 40 mg l^{-1}, and selection was started on day 14, 7 and 0 after protoplast isolation.

Hygromycin resistance testing

Hygromycin resistance of seedlings selected by PCR for *Ds* excision was tested by scoring for the rooting ability of *in vitro*-grown plants on MS30 supplemented with different concentrations of hygromycin (0, 10, 20, 30, 40, 60, 100 mg l^{-1}). Root formation was scored on day 10.

Rooting on MS30 supplemented with 40 mg l^{-1} hygromycin was used to test all regenerated shoots from the protoplast selection experiments as well as the control experiments. Regenerants with at least two growing roots were considered to be resistant; regenerants with no roots or with only one small, slow-growing root were considered to be sensitive.

Results

Development of transposon genotypes by crossing

To activate *Ds* linked to *P. infestans R1* resistance we crossed two of the four selected recombinants, Ds53-22 and -34 (Materials and methods) with TM17-2, a diploid potato clone susceptible to *P. infestans* and transformed with the *Ac* transposon-containing T-DNA construct pMK1GBSS*Ac* (Pereira *et al.*, 1991). TM17-2 contains one functional *Ac* that is transferred to progeny lines and was selected for *Ac* excisions early in development (Enckevort *et al.*, 2000b; Chapter 2). The progeny of these crosses, populations EE96-4311 (Ds53-22 X TM17-2; 18 seedlings) and EE96-4312 (Ds53-34 X TM17-2; 96 seedlings) were tested for segregation of kanamycin (100 mg l^{-1}), hygromycin (30 mg l^{-1}) and *P. infestans R1* resistances. Since both parental lines contained a kanamycin resistance gene, used as a selection marker during T-DNA transformation, the expected segregation for kanamycin resistance was 3:1; while *P.*

infestans R1 resistance, only present in the *Ds* parent, was expected to segregate 1:1. The observed segregation data for kanamycin resistance and *P. infestans R1* resistance are displayed in Table 1. Population EE96-4312 showed a distorted segregation for *R1* resistance ($\chi^2_{1:1} = 4.26$, $0.05 > P > 0.025$), while in both populations segregation for kanamycin resistance was as expected. Finally, 47 (8 and 39) kanamycin-resistant *R1* seedlings (KanR *R1*) were selected from the two populations (Table 1). None of the seedlings showed clear hygromycin resistance in the first rooting test on 30 mg l^{-1} hygromycin, indicating that *Ds* was not excised in the majority of the cells in these plants.

Table 1 Segregation data for kanamycin resistance versus susceptibility (KanR-KanS) and *P. infestans R1* resistance versus susceptibility (*R1-r*) for the populations EE96-4311 and EE96-4312

	KanR *R1*	KanS *R1*	KanR *r*	KanS *r*	Unknown	Total
EE96-4311	8	1	3	2	4	18
EE96-4312	39	8	14	15	20	96

The 47 KanR *R1* seedlings were further investigated by PCR to select *R1* resistant seedlings showing active *Ds* excision due to the presence of the introduced *Ac*. Using specific primers (Fig. 1), we determined the presence of empty donor sites (EDS-PCR) in 22 of the KanR *R1* seedlings as a 450-bp PCR product (data not shown).

The EDS-PCR data for a subset of plants were confirmed by Southern blot analyses (Fig. 2). Hybridisation with a NOS promoter probe revealed a *Pst*I restriction fragment of 4.0 kb corresponding to two copies of the original *Ds* T-DNA construct in the Ds53-22 parent and in 7 of the selected KanR *R1* seedlings from population EE96-4311. Complete excision of *Ds* will result in a bandshift from 4.0 kb to 2.3 kb. In the KanR *R1* seedling EE96-4311-12, a 2.3-kb *Pst*I fragment is clearly visible. This corresponds to an empty donor site, indicating complete *Ds* excision early in development. Two other KanR *R1* seedlings (EE96-4311-08 and -15) showed a faint 2.3-kb fragment, the intensity corresponding to about 10% EDS-containing cells. The *Pst*I restriction fragment of ± 3.5 kb in the TM17-2 parent reflects the presence of the pMK1GBSS*Ac* T-DNA construct in the *Ac* transposon-containing parent, which was inherited by the 3 plants showing an EDS fragment. The other 5 plants did not inherit

this *Ac* T-DNA and did not show active *Ds* excision. This same analysis was also performed for 16 of the 39 KanR *R1* seedlings selected from the EE96-4312 population. Nine seedlings were selected by EDS-PCR for *Ds* excision, which was confirmed by the presence of a faint 2.3-kb EDS fragment in Southern blot analyses (data not shown). The other 10 KanR *R1* seedlings with active *Ds* excision were selected from this population solely on the basis of the EDS-PCR. In total 22 (3 + 19) out of 47 KanR *R1* seedlings showed clear empty donor sites, indicating active *Ds* transposition in the presence of the *Ac*.

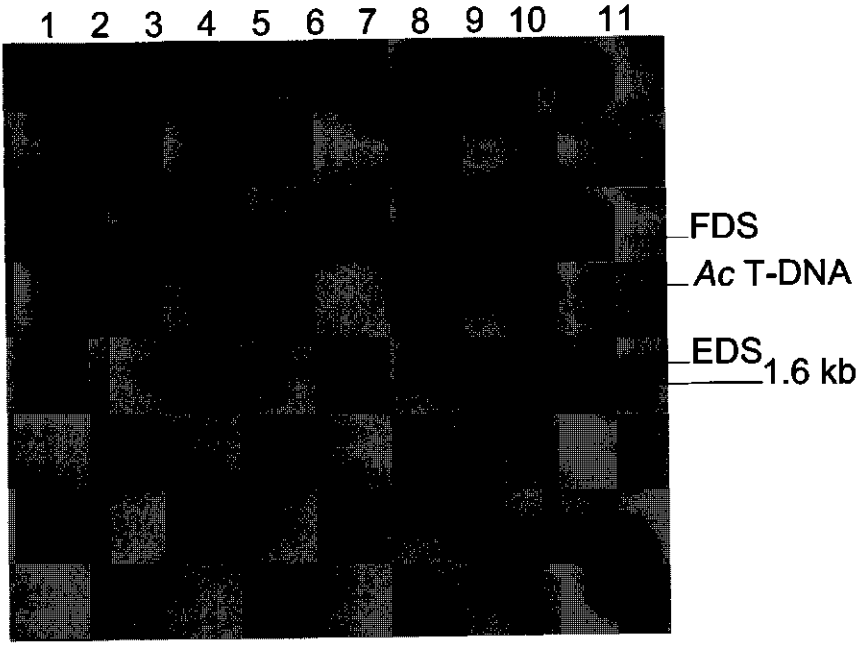

Fig. 2 *PstI* restriction of genomic DNA hybridised to NOS promoter probe to select for presence of the full donor site (*FDS*=4.0 kb), empty donor site (*EDS*=2.3 kb) and *Ac* T-DNA construct (3.5 kb) in Ds53-22 (*lane 1*), TM17-2 (*lane 2*) and 8 KanR *R1* seedlings EE96-4311-3 (*lane 3*), 6 (*lane 4*), 8 (*lane 5*), 9 (*lane 6*), 10 (*lane 7*), 12 (*lane 8*), 14 (*lane 9*) and 15 (*lane 10*). *Lane 11* 1-kb DNA size marker

To confirm that the EDS-PCR fragments were the result of *Ds* excision from the original T-DNA construct, we isolated the EDS-PCR fragments of 4 plants from an agarose gel and sequenced them. The DNA sequence of these four EDS-PCR fragments confirmed excision of *Ds* from the original T-DNA construct (Fig. 3).

Following selection of the 22 *R1* resistant seedlings showing active *Ds* excision (R1Ds/r-;Ac/-), as confirmed by PCR analyses, Southern blot analyses and EDS sequencing, the expression of hygromycin resistance was tested by rooting on MS30 supplemented with 10, 20, 30, 40, 60 and 100 mg l^{-1} hygromycin. Parental plant Ds53-22 and 2 of its R1Ds/r-;Ac/- progeny plants (EE96-4311-08 and -15) were only able to form roots on 10 mg l^{-1} hygromycin. EE96-4311-12 showed good rooting on 20, 30 and 40 mg l^{-1} hygromycin. The parental plant Ds53-34 and 18 of its R1Ds/r-;Ac/- progeny plants (EE96-4312-03, -05, -06, -14, -23, -27, -28, -30, -31, -37, -40, -43, -46, -52, -60, -63, -76, -89) showed good rooting on 10 and 20 mg l^{-1} hygromycin. EE96-4312-49 showed also some rooting on 30 mg l^{-1} hygromycin but not on 40 mg l^{-1} hygromycin. Despite the presence of empty donor sites shown by PCR and Southern blot analyses only 2 plants, EE96-4311-12 and EE96-4312-49, displayed an improved level of hygromycin resistance. In the case of plant EE96-4311-12 the complete EDS (absence of a full donor site; FDS), as shown on Southern blot (Fig. 2), explained the high level of hygromycin resistance. EE96-4312-49 did not show complete *Ds* excision on a Southern blot. These results suggest that *in vitro* rooting of shoots on 40 mg l^{-1} hygromycin can be used as a stringent criterion for complete *Ds* excision.

original site pHPT::*Ds*-Kan:	GT TGC GTG ACC	Ds	GCG TGA CCC GG
EE96-4311-12:	GT TGC GTG ...	-	... .. A CCC GG
EE96-4312-05:	GT TG.	-	... .. A CCC GG
	.. ..	-	... .. A CCC GG
EE96-4312-60:	GT TGC GTG AC.	-	... ... GG
EE96-4312-63:	GT TGC GTG ...	-	... .. A CCC GG

Fig. 3 Sequences flanking the *Ds* transposon in pHPT::*Ds*-Kan and the EDS sequences isolated from 4 R1Ds/r-;Ac/- seedlings displayed also in Table 2. The *underlined* sequence represents the target site duplication flanking the *Ds* insertion site

Protoplast isolation and selection of hygromycin-resistant regenerants

The 22 selected R1Ds/r-;Ac/- plants did display *Ds* excision at low levels, as shown by EDS-PCR and Southern blot analyses. Since only 1 seedling showed early and

complete excision of *Ds*, resulting in high levels of hygromycin resistance (HygR), it seems that most excision events occurred late in shoot development. It is expected that in these cases different excision events would result in different somatic sectors of *Ds* transposition. These sectors of *Ds* excision should contain HygR cells. The individual cells were separated using protoplast isolation to investigate whether this is the case. After regeneration of the protoplasts, all regenerated plants were individually tested for their hygromycin resistance. As negative controls the parental plants Ds53-22 and Ds53-34 and a progeny plant EE96-4312-21 (R1Ds/r-;-/-) were used.

Table 2 Number of calli, shoots and selected hygromycin-resistant (HygR) regenerants for parental genotypes Ds53-22 and Ds53-34, control genotype EE96-4312-21 (R1Ds/r-;-/-) and 22 selected R1Ds/r-;Ac/- genotypes from the seedling populations EE96-4311 and EE96-4312 after protoplast isolation and regeneration experiments with and without hygromycin selection

Genotype	No selection during protoplast regeneration			Hygromycin selection during protoplast regeneration		
	Calli	*Shoots*	*HygR*	*Calli*	*Shoots*	*HygR*
Ds53-22	10	0		47	0	
Ds53-34	100	45	0	134	1	0
EE96-4312-21	100	21	0	900	10	0
EE96-4311-08	0[a]			0[a]		
EE96-4311-12	100	49	22	1000	198	98
EE96-4311-15	300	82	11	800	160	101
EE96-4312-03	100	23	3	1000	166	83
EE96-4312-05	100	29	8	1000	198	121
EE96-4312-06	100	6[a]	2	1000	205	139
EE96-4312-14	100	70	15	1000	208	118
EE96-4312-23	100	51	2	1000	211	91
EE96-4312-27	10	0		10	0	
EE96-4312-28	100	47	7	1000	143	82
EE96-4312-30	100	0		419	0	
EE96-4312-31	100	30	2	570	21	4
EE96-4312-37	100	52	2	1000	248	92
EE96-4312-40	33	2	0	67	0	
EE96-4312-43	100	45	8	650	207	101
EE96-4312-46	14	9	0	103	0	
EE96-4312-49	100	48	3	1000	206	109
EE96-4312-52	3	3	0	1	1	1
EE96-4312-60	100	52	7	1000	203	93
EE96-4312-63	100	50	7	1000	203	130
EE96-4312-76	0			24	0	
EE96-4312-89	100	49	4	274	41	19
Total		691	103		2,619	1,382

[a] low due to infection.

Table 2 gives an overview of the protoplast regeneration data. The parental genotype Ds53-22 performed poorly in the protoplast experiments, and no regenerants were obtained at all. The other parental plant, Ds53-34, and the control EE96-4312-21 showed a better protoplast regeneration capacity. From the 22 selected R1Ds/r-;Ac/- plants 16 genotypes formed sufficient amounts of calli and 15 of them also showed shoot regeneration from these calli.

From parental clone Ds53-34, control EE96-4312-21 and selected R1Ds/r-;Ac/- seedlings a maximum of 100 calli were collected and at least 50 regenerating shoots were harvested when possible. All of the regenerated shoots were tested for their rooting ability on MS30 supplemented with 40 mg l^{-1} hygromycin (Table 2). The percentages of recovered hygromycin-resistant regenerants from the different genotypes are shown in Fig. 4. As expected, the parent Ds53-34 and the control EE96-4312-21 produced no HygR protoplast regenerants. EE96-4311-12 gave 45% HygR protoplast regenerants, suggesting early excisions. The other 14 R1Ds/r-;Ac/- plants that performed well showed a regeneration of 4-33% of HygR shoots (Table 2, Fig. 4). These data confirmed the presence of HygR cells, indicating excision of *Ds* from its original T-DNA location.

To select for cells with excision events represented by HygR protoplast regenerants at an earlier stage of the regeneration process, we added hygromycin during the protoplast culture starting at day 14 after isolation (see Materials and methods). From parent Ds53-34 and control EE96-4312-21 calli were obtained on hygromycin-containing medium but these rarely regenerated to shoots and they did not root on 40 mg l^{-1} hygromycin, confirming thereby the lack of *Ds* excision as expected. All 16 R1Ds/r-;Ac/- seedlings that performed well in protoplast culture were able to form relatively high numbers of actively growing calli on hygromycin-containing callus growth medium. Regenerated shoots were collected and tested for hygromycin (40 mg l^{-1}) resistance. Genotype EE96-4311-12 showed rooting for 50% of the regenerants (Table 2, Fig. 4). This percentage was comparable to the 45% HygR regenerants collected after protoplast regeneration without hygromycin selection and suggests that there is most likely an upper limit of real HygR regenerants that can be selected from this *Ds* excision locus. For all the other seedlings the use of hygromycin selection during callus culture and regeneration of shoots greatly increased the recovery of HygR regenerants to levels varying from 19% to 68% (Table 2, Fig. 4). These results indicate that *Ds* excision resulted in HygR cells in all of the selected R1Ds/r-;Ac/-

plants. On average, 53% of these protoplast regenerants were HygR in a rooting test on MS30 supplemented with 40 mg/l hygromycin. This is a 3.8-fold increase in HygR regenerants when compared to the average of 14% HygR regenerants obtained without using hygromycin selection in the tissue culture phase.

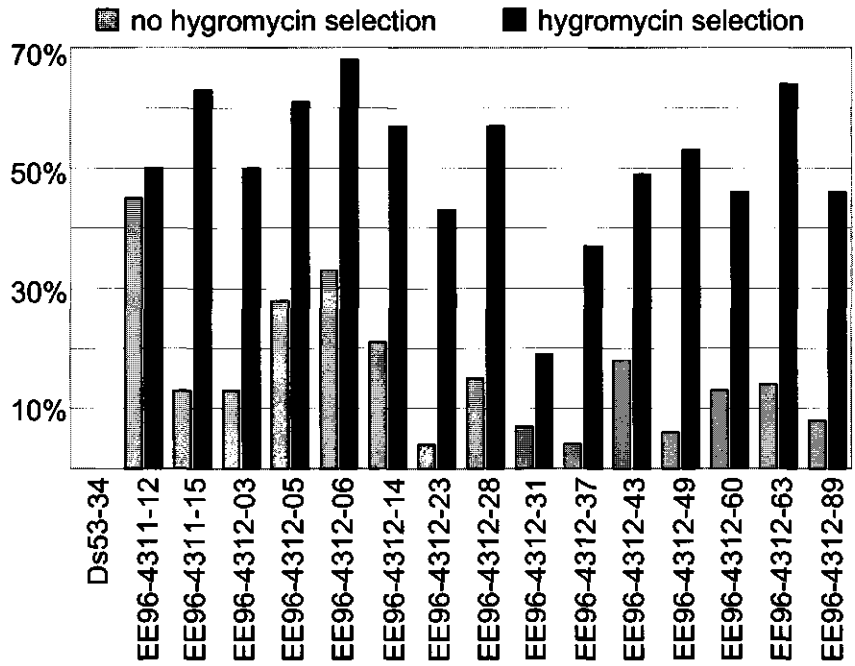

Fig. 4 Percentage hygromycin (40 mg l^{-1}) resistant protoplast regenerants obtained with and without hygromycin selection during protoplast culture

To further improve the frequency of HygR regenerant selection, we made the selection more stringent. Hygromycin was added starting on day 0, 7 or 14 after protoplast isolation, and the concentration of hygromycin in the culture media was increased to 30 or 40 mg l^{-1}. These more stringent conditions in the earlier stages of the protoplast culture process had a negative effect on the formation of calli and on the regeneration capacity of these calli into shoots. For only a few genotypes was it possible to collect reasonable amounts of calli and regenerating shoots (data not shown). After re-testing these shoots, the percentages of regenerants showing good rooting on 40 mg l^{-1} hygromycin had not increased indicating that the percentages

shown in Fig. 4 are the maximum numbers of HygR regenerants that can be obtained for the R1Ds/r-;Ac/- seedlings. In total 1,485 (103 + 1,382) HygR regenerants were selected from all R1Ds/r-;Ac/- seedlings used in the first protoplast regeneration experiment (Table 2). From later experiments with more stringent hygromycin selection 488 additional HygR regenerants were selected, making a total of 1,973 HygR protoplast regenerants available for further investigation.

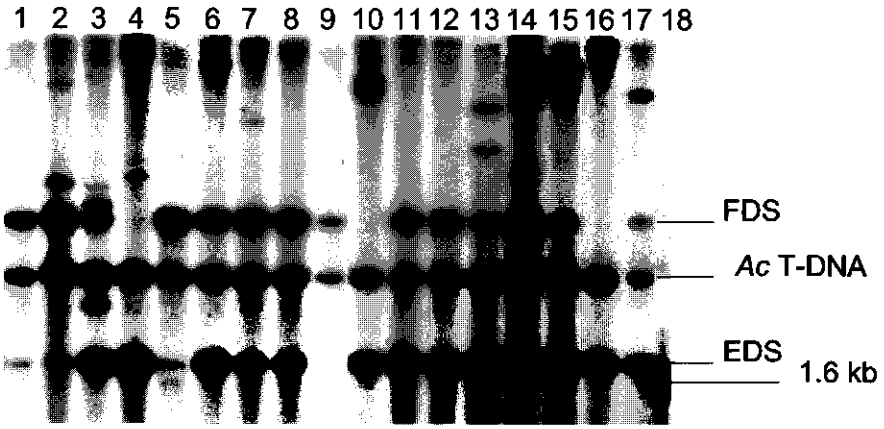

Fig. 5 *PstI* restriction of genomic DNA hybridised to NOS promoter probe to select for presence of the full donor site (*FDS* = 4.0 kb), empty donor site (*EDS* = 2.3 kb), *Ac* T-DNA construct (3.5 kb) and *Ds* re-insertion sites in the R1Ds/r-;Ac/- selected seedlings EE96-4311-37 (*lane 1*), EE96-4312-43 (*lane 5*) EE96-4312-49 (*lane 9*) and HygR protoplast regenerants from EE96-4311-37 (*lanes 2,3 and 4*), EE96-4312-43 (*lanes 6, 7 and 8*), EE96-4312-49 (*lanes 10, 11, 12 and 13*), EE96-4311-15 (*lanes 13, 15*), EE96-4312-05 (*lane 14*), EE96-4312-76 (*lane 16*) and EE96-4312-06 (*lane 17*). *Lane 18* the 1-kb DNA size marker

Molecular analysis of Ds excision and re-insertion

We performed Southern analysis on a subset of selected R1Ds/r1-;Ac/- seedlings and some of their HygR protoplast regenerants in order to analyse *Ds* excision in the HygR protoplast regenerants; some of these analyses are shown in Fig. 5. The R1Ds/r1-;Ac/- seedlings used for protoplast isolation all displayed two *PstI* fragments, 4.0- and 3.5-kb, corresponding to the *Ds* T-DNA construct and the *Ac* T-DNA construct,

respectively. Faintly visible fragments of 2.3 kb were also detected that correspond to a low amount of EDS fragments being present in these seedlings. All HygR protoplast regenerants showed a strong hybridising EDS fragment indicating the early or repeated excision of *Ds*, which in turn corresponds to the high level of hygromycin resistance for which these plants were selected. The original *Ds* parent accommodated two copies of *Ds*. Full donor site fragments were detected in most of the HygR protoplast regenerants, which indicates that one of the two *Ds*'s was not excised. Four plants (3 are shown in Fig. 5) showed complete EDS, indicating that in the starting protoplast of these plants both *Ds*'s were excised.

Most HygR protoplast regenerants showed clear *Ds* re-insertion fragments varying from one to eight new positions per individual HygR regenerant. Different regenerants from one single seedling showed different re-insertion patterns, indicating that they originated from independent transposition events. These data confirm the expectation that all selected HygR protoplast regenerants originated from independent excision sectors in the original R1Ds/r1-;Ac/- seedling.

Discussion

The efficient transposon tagging of specific genes can only be achieved when a large population of different insertional mutants can be produced easily. In plant species that produce large amounts of seeds, self- or testcross populations with a high germinal transmission frequency of transposon mutations can be used. However, attempts to develop efficient transposon tagging strategies in potato encountered the problem that at the tetraploid level this approach is rather difficult. After diploidisation the development of vigorous homozygous *Phytophthora infestans* resistant R1R1 plants turned out to be very time-consuming (unpublished). Therefore, an alternative method was developed to use the maize *Ac-Ds* transposable elements in a directed transposon tagging strategy for tagging the *R1* resistance gene. This strategy started with the selection of diploid potato plants heterozygous for the *R1* gene and having a *Ds* linked in coupling phase. By crossing these plants with a diploid potato containing an active *Ac* we were able to select R1Ds/r-;Ac/- seedlings showing *Ds* transposition in the somatic cells. These chimaeric seedlings were used in a protoplast regeneration approach to select cells with a *Ds* transposition event.

The transposase source used was from TM17-2, a diploid potato plant that contained an active autonomous *Ac* (Enckevort *et al.*, 2000b; Chapter 2). This plant was selected from a population of transformants showing various patterns of *Ac* transposition (Pereira *et al.*, 1991). These differences were probably due to a combination of position and dosage copy number effects. In the original transformant, TM17-2, early *Ac* excision occurred from one of the two copies of the pMK1GBSS*Ac* construct, resulting in two *Ac* homologous fragments. One of these appeared to be a complete *Ac*, while the other one (linked) had a deletion in the 3' site and was presumably not functional (Enckevort *et al.*, 2000b; Chapter 2). Crossing of TM17-2 with other diploid potato genotypes was expected to result in progeny having only one segregating active *Ac* transposase source. The heterogeneity in the sizes of the of excision sectors seen in the one- and two-copy transformants from the pMK1GBSS*Ac* population as described in Pereira *et al.* (1991) can, therefore, also be expected for progeny of TM17-2. Presumably these plants show a continuous expression of transposase activity throughout shoot development (Scofield *et al.*, 1992). The level of transposase enzyme activity is probably low because significant somatic variegation has already resulted from an extremely low level of transcription from the wild-type *Ac* promoter (Fußwinkel *et al.*, 1991).

In this study R1Ds/r-;Ac/- seedlings were selected by searching for EDS by PCR analyses. Southern blot analyses (Fig. 2) confirmed the presence of these EDS in somatic tissue. The DNA sequence of a few EDS-PCR fragments offered supplementary evidence for the excision of *Ds* from its original T-DNA construct (Fig. 3). Testing of these seedlings for rooting on hygromycin containing media showed that most of these plants did not display any hygromycin resistance, indicating that only a small number of the somatic cells contained excised *Ds* elements. This observation shows that *Ds* excision induced by the one-copy autonomous *Ac* transposase source seems to occur relatively late in development of the seedling, resulting in chimaeric plants with small sectors of independent excision events. Only 1 progeny seedling, EE96-4311-12 showed complete *Ds* excision early in development. Instead of looking further at the germinal transmission of these excision events, the chimaeric plants with an EDS site were considered as being an interesting pool of independent *Ds* transpositions.

Protoplast isolation and regeneration has proven to be an efficient method in potato to create a large progeny from an individual genotype. It was possible to

produce a large population of protoplast regenerants from our selected R1Ds/r-;Ac/- seedlings. The regenerated plants were tested for hygromycin resistance, which confirmed the excision of *Ds*. All plants that displayed an EDS-PCR fragment were able to produce HygR protoplast regenerants indicating that this selection criterion was accurate and that *Ds* excision from the T-DNA resulted in the expression of the hygromycin resistance gene. In experiments without hygromycin selection during the callus phase on average 14% of the regenerated plants were hygromycin-resistant, indicating that they were regenerated from somatic cells expressing *Ds* excision events. This percentage confirms the 10% EDS-containing cells estimated from the intensity levels of the EDS fragments on Southern blots. Hygromycin selection during protoplast regeneration made it possible to directly select for *Ds* excisions in protoplasts of the chimaeric R1Ds/r-;Ac/- plants. This resulted in a significant increase in the number of HygR protoplast regenerants selected - 53%, the upper limit of HygR regenerants that can be obtained from this *Ds* excision locus.

The somatic selection of *Ds* transpositions from individual cells facilitated the production of a large population of shoots with *Ds* excision events. Southern blot analysis confirmed the selection of independent *Ds* insertions as all investigated regenerants derived from one progeny plant showed different re-insertion sites of *Ds*, indicating independent excision and re-insertion. The occurrence of different re-insertion sites for *Ds* is an indication for the presence of small independent sectors with *Ds* transposition in the selected R1Ds/r-;Ac/- seedlings. Within the seedlings several independent *Ds* excisions and re-insertions probably occur due to the continuous expression of the *Ac* transposase throughout development.

In total, almost 2,000 HygR protoplast regenerants were selected that potentially represent 2,000 independent *Ds* insertions. This number of *Ds* transposon-induced mutations should theoretically be enough for the isolation of tagged mutants of the linked *R1* gene. Previously it was shown that in tomato the *Cf9* gene was tagged at a frequency of 1 in 1000 *Ds* transposition events from a position located at a distance of 3 cM (Jones *et al.*, 1994). In Arabidopsis, tagging of the *FAE1* gene by an *Ac* at a 22 cM distance resulted in 1 insertion from about 500-1,000 transpositions (James *et al.*, 1995). The somatic selection of *Ds* transposition and the rapid production of independent plants containing these transpositions as described here facilitate the production of large tagging populations needed for the transposon mutagenesis of

selected genes. This is particularly suitable for the mutagenesis of genes in heterozygous crops like potato.

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Chapter 4

The identification of potato *Phytophthora infestans R1* resistance response variants in a transposon-mutagenised population

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Abstract

Potato protoplast regenerants containing independent *Ds* transpositions were screened for an altered HR resistance reaction conferred by the *Phytophthora infestans R1* locus, using a detached leaf assay. The tagging population was derived from a *Ds* at chromosome 5 linked to the *P. infestans R1* locus. Transpositions were induced by *Ac* and selected using a hygromycin resistance marker for *Ds* excision. From 1973 hygromycin resistant protoplast regenerants 1564 plants could be screened for their *R1* resistance reaction yielding 33 plants with a potential altered response reaction. After *in vitro* propagation, the HR resistance reaction was quantified for the selected variants and their parental seedlings. This disclosed four regenerants with a clearly altered *R1* resistance phenotype with less than 50% of the inoculated leaves showing the *R1* type HR response. In these putative mutants clear colonisation and sporulation of *P. infestans* indicated that the *R1* gene action was not fully functional. Flanking sequences of the *Ds* insertions in two mutants showed similarity to genes described in public databases with predicted proteins that have a potential biological role in disease resistance. These results validate the screening of the transposon-mutagenised population for the identification of putative HR signalling mutants. The corresponding genes could be useful in further elucidation of the *R1* resistance mediated signalling pathway leading to HR in potato.

Introduction

The oomycete *Phytophthora infestans* (Mont.) de Bary is worldwide the main pathogen of the potato crop causing late blight. Early breeding for resistance resulted in the introduction of genes from the Mexican wild species *Solanum demissum* into the cultivated potato (Umaerus *et al.*, 1983; Wastie, 1991), but newly occurring virulent races of *P. infestans* circumvented these *R* gene mediated resistances. Characteristic for *R* gene mediated resistance reactions is the hypersensitive response (HR) leading to local cell death causing necrotic spots at the site of attempted infection. Genetic analysis showed that activation of HR is highly specific and induced upon recognition by a specific *R* gene product and a corresponding avirulence gene product in the pathogen (Black *et al.*, 1953; Flor, 1942; Hammond-Kosack and Jones, 1997).

From earlier breeding studies it is known that the dominant *R* gene mediated resistance reactions from wild *Solanum* species can show partial resistance or an intermediate HR response after backcrossing to *S. tuberosum* (Graham, 1963; McKee, 1962; Toxopeus, 1958; 1959). The *R* gene mediated HR lesions can vary in size depending on the backcross parent used, indicating that the major dominant *R* gene resistance reaction is influenced by other genes. QTL mapping in *S. tuberosum* populations segregating for partial *P. infestans* resistance, identified 19 QTLs on 13 chromosomal regions (Leonards-Schippers *et al.*, 1994). One QTL was detected on chromosome 5 in the region of the *P. infestans* resistance locus *R1* (Leonards-Schippers *et al.*, 1992) and linked to QTLs for maturity and vigour (Collins *et al.*, 1999; Oberhagemann *et al.*, 1999). These QTLs on chromosome 5 very likely represent minor genes that play a role in both *R* gene mediated HR resistance responses and developmental processes which indirectly influence the resistance response. In this potato chromosome 5 region several other resistance loci with specificity to different pathogens like the PVX virus (Jong *et al.*, 1997; Ritter *et al.*, 1991) and the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* (Kreike *et al.*, 1994; Rouppe van der Voort *et al.*, 1998) are identified.

In recent years a large number of resistance genes (reviewed in Hammond-Kosack and Jones, 1997) involved in the HR reaction of different plant-pathogen interactions have been isolated. One structural motif shared by almost all isolated resistance genes is the leucine-rich repeat (LRR), which is found to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994; Leckie *et al.*, 1999) that may

interact with the avirulence gene product, triggering a pathway that eventually leads to HR. Another class of resistance genes are the receptor-like protein kinases identified in tomato (*Pto* and *Pti*; Martin *et al.*, 1993; Zhou *et al.*, 1995) and rice (*Xa21*; Song *et al.*, 1995) that probably play an important role in activating the downstream signalling by phosphorylation after recognition between the R protein and the Avr product (Tang *et al.*, 1996).

To identify genes acting in the *R1* type HR resistance signal transduction pathway in potato, upon infection with a virulent *P. infestans* race, a transposon tagging approach was chosen for the selection of insertion mutants. Transposon tagging has been successfully used for the isolation of disease resistance *R* genes from self-fertilising crops, like tomato, tobacco and flax (Jones *et al.*, 1994; Lawrence *et al.*, 1995; Whitham *et al.*, 1994). A two component *Ac-Ds* based transposon tagging population in diploid self-incompatible potato genotypes heterozygous for the *P. infestans R1* resistance gene was developed (Enckevort *et al.*, 2000a; Chapter 3) with a linked *Ds* transposon on chromosome 5 (18 cM) exhibiting active transposition. Protoplast regeneration and hygromycin resistance based selection for *Ds* excision in somatic tissue enabled the production of a large population of regenerants, heterozygous for the *R1* gene and with predominantly independent *Ds* insertions. The current report describes the outcome of the screening for *P. infestans R1* resistance variants. After a primary screening, a number of potential variants with an altered *P. infestans R1* resistance response were identified. A second screening on replicate samples allowed a quantification of the *R1* type HR resistance response on parental plants and their regenerants. A subset of plants with a distinctly altered *R1* resistance response were identified and described, both phenotypically and molecularly.

Materials and methods

Generation of the transposon tagged population

The production of the tagging population used in this study was described previously (Enckevort *et al.*, 2000a; Chapter 3). A *P. infestans R1* resistant diploid potato clone was transformed with a *Ds*-transposon containing T-DNA construct (El-Kharbotly *et al.*, 1995). Genotypes with close linkage (18 cM) between the *R1* gene and a *Ds* T-DNA

on chromosome 5 were selected (El-Kharbotly *et al.*, 1996a) and two of these (Ds53-22 and Ds53-34) were crossed with the *Ac* transposon containing diploid potato clone TM17-2. In total 22 *R1* seedlings with active *Ds* transposition (R1Ds/r-;Ac/-; EE96-4311-08, -12, -15 and EE96-4312-03, -05, -06, -14, -23, -27, -28, -30, -31, -37, -40, -43, -46, -49, -52, -60, -63, -76, -89) that exhibited about 10% somatic transposition were selected. Protoplast isolation and selection for hygromycin resistance enabled the direct selection of *Ds* excision events from 18 of these seedlings. Hygromycin resistant protoplast regenerants (HRPR's) with predominantly new independent *Ds* insertion sites were selected.

Propagation of HRPR's

The selected HRPR's were grown *in vitro* on MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose (MS30) and 40 mg l^{-1} hygromycin. After two weeks these HRPR's were transferred directly to small pots in the greenhouse in parallel with the susceptible parental genotype TM17-2 and the resistant parental genotypes Ds53-22 and Ds53-34. The selected HRPR's were transferred in small groups to spread the resistance screening over a period of time and after 6 to 10 weeks fully developed leaves were collected for *P. infestans* resistance screening. The tested plants continued to grow till tuber formation and harvesting, allowing additional screenings with newly formed leaves.

For ploidy determination, samples were harvested of fresh greenhouse grown leaves and flow cytometric analysis for the DNA content was carried out (Laat *et al.*, 1987).

P. infestans R1 gene resistance testing

P. infestans R1 resistance tests were performed as described previously (El-Kharbotly *et al.*, 1994), using *P. infestans* 89148-09 (race 0, kindly supplied by Dr. F. Govers, Laboratory of Phytopathology, Wageningen University and Research Centre, The Netherlands). For growth and maintenance rye agar medium supplemented with 20 mg l^{-1} sucrose was used (Caten and Jinks, 1968). To perform large-scale leaf inoculation

with *P. infestans* sporangiospores, rye-agar-grown race 0 was washed with 10-15 ml cold tap water (4°C) and the resulting sporangiospore suspension used to inoculate 10 Bintje tuber slices (1 cm thickness). After 5 days newly formed sporangiospores were again washed and the sporangiospore solution used to inoculate 20-50 tuber slices of Bintje in order to obtain 1-2 l of sporangiospore solution. This solution was checked for the presence of at least 2,000 spores ml^{-1}, though usually the amount of spores was 5,000 spores ml^{-1} or more.

After 6 - 10 weeks growth in the greenhouse two leaves of each plant were harvested, placed in columns of water absorbent substrate, and put in containers (46 x 31 x 8 cm) closed with transparent covers. In each container 10 plants represented by 2 leaves each and one leaf of the susceptible control cultivar Bintje or TM17-2 were tested. In one screening experiment 15 - 30 containers were used so that 150-300 plants could be tested in parallel. In each experiment always Ds53-22, Ds53-34 and TM17-2 were tested as additional controls. Each leaf in the experiments was sprayed with about 5 to 10 ml of the sporangiospore solution. The estimated number of sporangiospores inoculated per leaf was 10.000 to 50.000. After 5 days in high humidity at 16°C, all leaves were evaluated for the development of *P. infestans* infection symptoms. At day 6 a second evaluation for disease symptoms on all leaves was performed. When development of symptoms occurred the leaves were kept for an additional 2 days to study disease development in more detail using a binocular.

In vitro propagation of P. infestans R1 resistance variants

R1 resistance variants selected in the first *P. infestans* resistance screening were transferred from the greenhouse to *in vitro* for preservation. The stem from which the *P. infestans* inoculated leaves were harvested was cut in pieces, sterilised 1 minute in 70% ethanol and 20 minutes in 1.5% hypochlorite followed by 3 washes of 10 minutes in sterile water. Brown stem pieces were removed and individual axillary buds were put on MS30 for development. After 1 to 2 weeks one or a few *in vitro* developed shoots were removed from the old stem material and transferred to fresh medium for preservation. *In vitro* 10 or 35 cuttings of each variant were produced and these were transferred to the greenhouse for re-testing of the *P. infestans R1* resistance.

Table 1 Primary screening of HRPR's for the selection of variants with an altered *R1* type HR resistance response. The parental genotypes and the HRPR variant distribution are outlined

R1Ds/r-;Ac/- Seedling	total # selected HRPR's	# HRPR's tested with *P. infestans* race 0	# HRPR's Variant	Variant plant #	Ploidy level
EE96-4311-12	126	72	1	702	4x
EE96-4311-15	112	81	2	35	2x
				994	2x
EE96-4312-03	86	63	2	1515*	2x
				1921	nd
EE96-4312-05	129	84	2	836*	4x
				842	4x
EE96-4312-06	243	188			
EE96-4312-14	195	168	2	925	4x
				1587	2x
EE96-4312-23	93	82			
EE96-4312-27	5	0			
EE96-4312-28	89	71	7	487*	2x
				998	2x
				999	4x
				1000	2x
				1001	4x
				1005	4x
				1357	2x
EE96-4312-31	7	4			
EE96-4312-37	134	120	4	151*	4x
				510*	4x
				524	2x
				551	4x
EE96-4312-43	109	91	6	570*	4x
				688*	4x
				1528	4x
				561	x-4x
				562	x-4x
				574	4x
EE96-4312-49	152	111	6	600	4x
				601	4x
				633*	2x
				1050	2x
				1055*	4x
				1073	4x
EE96-4312-52	1	1			
EE96-4312-60	134	112	1	667	4x
EE96-4312-63	168	155			
EE96-4312-76	83	65			
EE96-4312-89	107	96			
Total	1973	1564	33		

= number, nd = not determined, * variants with confirmed susceptible reaction after re-inoculation

Inverse PCR

To determine the sequence of the sites of *Ds* insertion, flanking DNA of *Ds* was isolated by inverse PCR (IPCR; Triglia *et al.*, 1988). Plant genomic DNA, isolated from greenhouse-grown leaves (Pereira and Aarts, 1998), was restricted with *Hae*III, self-ligated and restricted with *Bam*HI and *Bgl*II. *Bgl*II restriction prevents the amplification of the *Ds* transposon flanking sequences in the original T-DNA construct. Primer 5' cgg gat gat ccc gtt tcg tt (*Ac* position 197-216) and primer 5' gat aac ggt cgg tac ggg at (*Ac* position 44 - 35) were used to amplify the 5' *Ds/Ac* flanking sequences. After a hot start (10 min 94°C), 35 PCR cycles (1 min 94°C, 1 min 60°C, 2 min 72°C) resulted in the amplification of *Ac* and *Ds* 5' flanking sequences. IPCR products were separated on a 1% TBE agarose gel to determine number and size of fragments. After phenol::chloroform extraction and isopropanol precipitation, the IPCR products were cloned in pGEM ¯T easy vector (Promega Corporation). For each sample three clones were sequenced using an automated ABI 373 DNA sequencer. The obtained *Ds* flanking sequences were compared to known sequences by BlastN and BlastX homology searches in the public databases (Altschul *et al.*, 1997).

Results

Primary screen for R1 type HR resistance variants in the Ds tagged population

The *Ds* tagged potato population of 1973 hygromycin resistant protoplast regenerants (HRPR's) was derived from 18 heterozygote *R1* resistant seedlings with active *Ds* transposition (R1Ds/r-;Ac/-, Table 1). The ploidy level of a sample of 76 HRPR's revealed 31 diploid, one hexaploid (6x), two mixoploid (1x-4x) and the rest tetraploid plants due to the protoplast regeneration. Morphological variation in plant type due to somaclonal variation was visible but 1564 HRPR's developed well in the greenhouse to harvest leaves for the *P. infestans* inoculation experiments.

We devised a detached leaf assay where *P. infestans* race 0 infection was studied, starting on day 5, on each individual HRPR leaf and compared to the susceptible parent TM17-2 and the resistant parents Ds53-22 and Ds53-34. The susceptible parent and the susceptible control cultivar Bintje always showed distinctive

colonisation and abundant sporulation on day 5 or 6 (Fig. 1). In contrast the resistant parents Ds53-22 and Ds53-34 always displayed characteristic necrotic HR spots upon infection and this *R1* type HR reaction was observed for most of the analysed HRPR's. The phenotype of HRPR 836 was distinctly susceptible with colonisation and sporulation over large leaf areas (Fig. 1). Other HRPR's sometimes showed larger necrotic regions indicating colonisation of the leaves (Fig. 1). When this colonisation resulted in sporulation (Fig. 1) the HRPR was selected as a potentially susceptible *R1* variant, although necrotic spots were additionally visible on the green parts (Fig. 1) indicating at least a partial HR activation. In this first round of screening 33 putative susceptible variants, derived from 10 R1Ds/r-;Ac/- seedlings were selected (Table 1). Re-inoculation tests of newly grown leaves of the selected variants confirmed the susceptible reactions for 9 variants.

Fig. 1 Reaction phenotypes observed on different genotypes after inoculation of detached leaves with *P. infestans* race 0. a) TM17-2, susceptible parent; b) detail of sporulation on TM17-2; c) HRPR 836; d) HRPR 1587 showing both the *R1* type HR response and necrotic regions with sporulation; e) detail of HR spot on HRPR 1587; f) detail of sporulation on the necrotic region of HRPR 1587; g) necrotic regions on R1Ds/r-;Ac/- seedling EE96-4312-03, minor sporulation was detected in such regions; h) clear colonisation on variant 1000; i) detail of sporulation on variant 1000

Secondary R1 resistance screen of parental seedlings and the selected variants

Since some selected variants displayed a convincing susceptible phenotype a re-evaluation for the *P. infestans* resistance reaction was performed on a part of the selected variants and their parental seedlings. From the first set of 33 *R1* variants, ploidy level analysis enabled the identification of plants with chromosomal anomalies that were potentially somaclonal variants, and selection of a set of diploids. All the diploid variants together with those with a reproducible susceptible phenotype were used for this detailed phenotypic analysis. Parallel to this analysis also 9 parental seedlings were tested for their *R1* type HR resistance reaction. By *in vitro* propagation of selected variants and their parental seedlings multiple cuttings useful for a quantitative analysis of the resistance reaction were obtained. After *P. infestans* inoculation on two leaves of each plant, the developing symptoms were carefully evaluated and followed in time using a binocular when necessary to detect sporulation (Table 2).

The *R1* resistant parental plant Ds53-34 always showed the complete *R1* type HR response, with small necrotic spots on inoculated leaves. The *R1* resistant progeny of Ds53-22 and Ds53-34 (EE96-4311-15 and EE96-4312-03, 05, 14, 28, 37, 43, 49 and 76) displayed an intermediate resistance phenotype (Table 2). With the exception of seedling EE96-4312-76, all other seedlings showed on several leaves (6-35%) necrotic spots that developed into necrotic regions covering 5 to 20% of the leaf area (Fig. 1). By using a binocular, little sporulation was observed in these regions indicating minor escape of *P. infestans* from the normal *R1* type HR response. In a few leaves the necrotic region covered almost 100% of the leaf area and colonisation with sporulation was observed indicating susceptibility of the leaf and escape from the *R1* type HR resistance response. Seedling EE96-4312-14 showed in this analysis only in 47% of the leaves a clear *R1* type HR response. However, from the 168 HRPR's derived from this seedling and tested in the first screening for *R1* resistances only 2 were selected as putative variants (Table 1). This indicates that the intermediate phenotype for this and other parental seedlings did not result in an overestimation of putative variants in the first screening. This quantification of the resistance response using replicate analysis of a single seedling is, however, needed to identify putative mutants in the *Ds* tagging population derived from these seedlings.

Table 2 Qualitative and quantitative analysis of the resistance reaction and disease development on selected variants compared to parental controls

	# cuttings	% leaves with HR	% leaves with maximal 20% of leaf area necrotic with some sporulation	% leaves with 20 to 100% leaf area necrotic with colonisation and sporulation (max % leaf area covered)		% Dead (Rotten)
Parents						
Ds53-34	10	100	0	0		0
TM17-2	10	0	0	100	(100)	0
R1Ds/r-;Ac/- Seedlings						
EE96-4312-76	10	100	0	0		0
EE96-4312-43	9	94	6	0		0
EE96-4312-37	19	87	13	0		0
EE96-4312-28	20	75	23	2	(35)	0
EE96-4312-49	10	75	20	5	(25)	0
EE96-4312-05	20	75	15	5	(40)	5
EE96-4312-03	21	71	19	10	(60)	0
EE96-4311-15	20	60	35	5	(50)	0
EE96-4312-14	19	47	18	32	(100)	3
Variants						
EE96-4312-43 570	9	100	0	0		0
688	7	100	0	0		0
EE96-4312-37 510	10	95	5	0		0
524	10	90	5	0		5
EE96-4312-28 487	35	41	43	16	(100)	0
998	34	60	38	2	(35)	0
1000	35	16	34	50	(100)	0
1357	34	63	24	9	(70)	4
EE96-4312-49 601	10	60	30	10	(60)	0
633	10	70	25	5	(60)	0
1050	9	78	17	5	(45)	0
1055	10	80	10	5	(50)	5
EE96-4312-05 836	34	7	12	68	(100)	13
EE96-4312-03 1515	31	76	16	8	(50)	0
EE96-4311-15 35	14	82	0	7	(50)	11
994	8	44	0	56	(100)	0
EE96-4312-14 1587	1	50	0	50	(60)	0

= number, HR = hypersensitive response

The re-evaluation of the resistance response reaction for the variants 487, 1000, 836 and 994 showed a clear deviation in phenotype when compared to the parental seedlings. In variant 487 the *R1* type HR resistance response was clearly detected in only 41% of the inoculated leaves. On 16% of the leaves necrotic regions covered over 20% of the leaf area and colonisation and sporulation was detected indicating an alteration to a putative susceptible *R1* variant. Variant 1000 showed colonisation and sporulation on 50% of the leaves and this clearly resembled the TM17-2 *P. infestans* susceptible parental phenotype (Fig. 1). Only 16% of the variant 1000 leaves showed the normal *R1* type HR resistance response. The distinctive susceptible phenotype of variant 836 in the first screening of the HRPR population (Fig. 1) was repeatable in this analyses but quick senescing of the leaves of this variant made the final analysis difficult. Softening and rotting interfered with the evaluation of the disease development. Variant 994 showed a striking phenotype when analysed on the *in vitro* propagated cuttings. From every cutting the youngest leaf showed colonisation with sporulation, always combined with leaf softening and rotting. The second oldest leaf analysed always showed a normal *R1* type HR resistance phenotype.

Re-evaluation of the resistance phenotype of the variants 570, 688, 510, 524, 633, 1050, 1055, 1515 and 35 did not show any difference when compared quantitatively to the parental seedlings. Variants 998, 1357 and 601 did display a minor deviation from the resistance phenotype of the parental seedlings they were derived from. Relatively high amounts of leaves with necrotic regions and sporulation were observed indicating probably why they were selected as putative *R1* mutants in the first screening. Still these variants were not completely susceptible, as the necrotic sporulating regions were restricted to about 20% of the leaf area. Only 2 to 10% of the leaves, like in the parental seedlings, showed larger necrotic regions with colonisation and minor sporulation. These variants were not regarded as potential *Ds* insertion mutants for the *R1* resistance reaction.

Analysis of Ds insertion site flanking sequences

Putative *P. infestans* resistance variants were analysed molecularly to determine whether the sequence tagged by the *Ds* transposon could be correlated to the

increased susceptibility reaction. *Ds* flanking sequences isolated by inverse PCR (IPCR) from 14 variants, seedling EE96-4312-43, parent Ds53-34 and parent TM17-2 were cloned, sequenced (average 156 bp) and compared to sequences in the public databases using BlastN and BlastX homology searches (Table 3).

A similarity search using BlastN resulted in significant hits in the database for 5 sequences derived from four variants and one parental seedling. Two identical hits to the T-DNA vector were identified for variant 1587 and variant 570. Since these two variants were not regenerated from the same seedling and two independent *Ds* insertions on an identical position are unlikely to occur, it was assumed that this insertion was an *Ac* insertion in the *Ac* T-DNA vector inherited from the TM17-2 parent. This indicated re-insertion of *Ac* to very closely linked positions. In seedling EE96-4312-43 an *Ac* or *Ds* insertion in an *Ac* sequence was identified. In variant 688 the insertion sequence was homologous to a *S. tuberosum* specific sequence in the non-coding region between two U1snRNA genes (U1-3 and U1-4; Vaux *et al.*, 1992). The flanking sequence of the *Ds* insertion isolated from variant 994 showed homology to two tomato genes: a tomato abscission polygalacturonase (*TAPG2*; Hong and Tucker, 1998) and a tomato anionic peroxidase (*TAP1*; Roberts and Kolattukudy, 1989). The potato sequence was homologous to a conserved 300 bp imperfect inverted repeat (TAPIR) found in the 5' upstream sequence of *TAPG2* and in the first intron of *TAP1* (Hong and Tucker, 1998).

A search for similar proteins in the public database using BlastX identified three additional significant hits. In the *Ac* parent TM17-2 the *Ac* flanking sequence of 141 bp showed 58% identity and 80% conserved homology to the nitrite transporter gene identified in *Cucumis sativus* (NCBI Accession number CAA93316). This same *Ac* insertion was also identified in variant 524. These data indicate the inheritance of two independent *Ac* positions from TM17-2 in progeny, one in the cloning vector (described above) and one in a nitrite transporter homologous sequence.

For variant 1000 the BlastX similarity search for two different *Ds* insertions identified homology to a leucine rich repeat containing protein kinase from *Oryza longistaminata* (Tarchini *et al.*, 2000) and a receptor protein kinase-like protein from *Arabidopsis thaliana* (BAC clone F13I12). These sequences were both identified due to their homology to the serine/threonine kinase domain of the *Xanthomonas* resistance gene *Xa21* isolated from *O. longistaminata* (Song *et al.*, 1995).

Table 3 Summary of *Ds* flanking DNA of putative mutants compared to public databases. Similarity to known DNA sequences (BLastN) and proteins (BlastX) with significance levels are indicated

Plant	Size[1]	BlastN	E[2]	BlastX	E
Ds53-34	139	ns		ns	
TM17-2	141	ns		nitrite transporter *Cucumis*	$3\ e^{-8}$
	118	ns		*sativus*	
				ns	
EE96-	162	ns		no hits found	
4312-43	36	Zea mays *Ac*	$6\ e^{-12}$	no hits found	
1587	146	cloning vector	$6\ e^{-69}$	Beta-lactamase	$1\ e^{-15}$
487	192	ns		ns	
	100	ns		ns	
998	117	ns		ns	
1000	289	ns		receptor protein kinase-like protein *Arabidopsis thaliana*	$9.5\ e^{-2}$
	496	ns		leucine rich repeat containing protein kinase *Oryza longistaminata*	$2\ e^{-7}$
1357	31	ns		no hits found	
524	141	ns		nitrite transporter (idem TM17-2) *Cucumis sativus*	$8\ e^{-8}$
570	138	ns		no hits found	
	146	cloning vector (idem 1587)	$2\ e^{-71}$	Beta-lactamase	$4\ e^{-14}$
688	256	ns		ns	
	30	*S. tuberosum* U1snRNA between U1-3 and U1-4	e^{-3}	no hits found	
633	76	ns		no hits found	
1055	125	ns		no hits found	
35	206	ns		ns	
836	92	ns		no hits found	
994	399	- polygalacturonase 2 (*TAPG2*) *Lycopersicon esculentum*	e^{-32}	no hits found	
		- *TAP1* for anionic peroxidase *Lycopersicon esculentum*	$6\ e^{-19}$		
1438	22	ns		no hits found	

ns = no significant homology; [1]Size in basepairs of isolated DNA sequence that flank the *Ds* inserts; [2]Expect-value in BLAST search reporting significance of similarity

Discussion

Selection of P. infestans R1 resistance variants

A method was developed using somatic insertion mutagenesis and *in vitro* selection for the identification of transposition events in diploid potato heterozygous for the *R1* gene (Enckevort *et al.*, 2000a; Chapter 3). Protoplast regeneration combined with a stringent

hygromycin selection for *Ds* transposition resulted in the development of a tagging population. For testing the *Ds* transposon tagged population to identify variants in the *P. infestans R1* resistance reaction, a slightly modified detached leaf assay was employed. This test should be able to distinguish qualitative changes in the *R1* type HR reaction as well as quantitative differences in the resistance responses (Vleeshouwers *et al.*, 1999). The first screening in the population of *Ds* insertional mutants yielded 33 putative variants for the *P. infestans R1* resistance phenotype. However, the reproducibility of this first analysis was low for several genotypes and the observed altered phenotypes usually showed a relatively small deviation from the partial *R1* type HR resistance response. Therefore, a clearly susceptible *R1* mutant was not found.

Many mutant screens in Arabidosis have identified genes required for expression of resistance which are candidate signal transduction genes like *NDR1* (Century *et al.*, 1995), *EDS1* (Parker *et al.*, 1996), *PAD1*, *PAD2*, *PAD3* and *PAD4* (Glazebrook *et al.*, 1996) and *PBS1*, *PBS2* and *PBS3* (Warren *et al.*, 1999). Most of these mutations affect the function of a subset of *R* genes tested (Aarts *et al.*, 1998; Warren *et al.*, 1999). Genes have also been identified that are required for the barley powdery mildew mediated *Mla-12* resistance (*Rar1* and *Rar2*; Peterhänsel *et al.*, 1997; Torp and Jørgensen, 1986), for the tomato *Pseudomonas syringae* pv *tomato* resistance gene *Pto* (*Prf*; Salmeron *et al.*, 1994; 1996) and *Pti*; (Zhou *et al.*, 1995; 1997) and for the tomato *Cf-9* (*Rcr-1* and *Rcr-2;* Hammond-Kosack and Jones, 1994) and *Cf-2* mediated *Cladosporium fulvum* resistance reactions (*Rcr-3;* Dixon *et al.*, 2000). Although the identified mutations in Arabidopsis, barley and tomato reduce resistance conferred by one or several specific *R* genes, most do not completely eliminate the defence responses. The identification of these partly susceptible mutations was only possible using clear selection methods like screening for loss of a specific defence response (Glazebrook *et al.*, 1996), screening for loss of defence gene induction (Cao *et al.*, 1994) or using the yeast two-hybrid system (Zhou *et al.*, 1997).

In a primary screen for *R1* mutants all deviations from the *R1* type HR resistance response were recorded. In the second year using replicated clones, a subset of selected HRPR variants was re-tested for their *R1* type HR reaction and their phenotype compared to that of their parental seedlings. Within a single *P. infestans* inoculation experiment, the *R1* type HR resistance was quantified and a quantitative

comparison between variants and the parental seedlings was made. For 12 variants this repeated testing showed that the phenotype was not significantly different from the parental seedling. In these seedlings the major dominant *R* gene resistance reaction is quantitatively influenced by minor *S. tuberosum* or *S. demissum* genes (Graham, 1963; McKee, 1962; Toxopeus, 1958; 1959). The observed variation in the phenotype of the selected variants is probably due to the partial resistance phenotype and not related to a transposon-induced mutation of the *R1* gene.

Finally the more quantitative screening revealed four variants (487, 1000, 836 and 994) displaying significantly higher levels of susceptibility to *P. infestans* race 0 when compared to the phenotype of the parental seedlings.

Potential biological correlation between Ds tagged sequence and variant phenotype

Variant 487 showed a relatively minor deviation from the parental seedling phenotype and the *Ds* tagged site showed no significant homology in the public databases. The observed phenotype is probably related to random or somaclonal variation.

Variant 1000 displayed a much more susceptible phenotype: colonisation and sporulation were observed in 50% of the inoculated leaves. Lesser necrosis, indicating absence of the *R1* type HR response, conferred in many inoculated leaves of this variant a phenotype similar to the susceptible parent TM17-2. Both of the flanking sequences of the two identified *Ds* insertions in this variant showed homology to serine/threonine protein kinases. Although the protein identity compared to *Xa21* (Song *et al.*, 1995) is less than 50%, the intron identified in the *Xa21* kinase domain was also present in the 289-bp potato sequence at exactly the same nucleotide position. Therefore, the conclusion is that the *Ds* insertions in this variant resulted in the mutation of two *Xa21* homologous kinase genes that are probably functional in the signal transduction pathway leading to the *R1* type HR resistance reaction in potato.

Variant 836 was selected in the primary resistance screening but latter analyses showed early senescence of leaves. After inoculation with *P. infestans* race 0, a necrotic HR response was observed on day 5 but later *P. infestans* was able to escape and colonised the complete leaf finally resulting in rotting. Probably the early senescence made the inoculated leaves more susceptible to *P. infestans*. Early maturation is known to increase the susceptibility of leaves of both susceptible and

resistant potato plants (Grainger, 1956; Lowings and Acha, 1959). The *Ds* flanking sequence isolated from this variant showed no homology in the databases, either a yet unidentified transposon mutation or a somaclonal variation resulted in the early senescence of the detached leaves. This variant is the only tetraploid variant with a clear susceptible phenotype. Therefore, in general the tetraploidisation was not a disturbing factor for the phenotypic analysis in the tagging population.

Variant 994 showed the same change in softening and rotting of detached leaves as described for variant 836, but only relatively young leaves exhibited this phenotype. The older fully developed fresh leaves expressed a clear *R1* type HR resistance response. It is known that very young leaves are more susceptible to *P. infestans* than fully developed mature leaves (Grainger, 1956; Lowings and Acha, 1959; Umaerus, 1969). In variant 994 *Ds* is integrated near a region in potato homologous to the TAPIR region in the tomato defence related genes *TAPG2* and *TAP1*. In these TAPIR regions auxin and abscicic acid *cis*-response elements were identified (Hong and Tucker, 1998). Increasing the distance between such a *cis*-regulatory region and the promoter due to a *Ds* insertion could create a mutation or influence the expression level of the gene involved. Tomato polygalacturonase genes (*TAPG*) are involved in fruit ripening and cell wall degrading processes. Possibly due to a transposon mutation in a potato polygalacturonase homolog, the polygalacturonase activity in variant 994 might be reduced and, therefore, development to maturity might be delayed. Similarly, mutations of an anionic peroxidase might also influence development. This could explain the occurrence of the susceptible phenotype for the young leaves of variant 994, while the older leaves were fully developed and displayed the *R1* type HR resistance reaction entirely.

Candidate components for the R1 resistance pathway

The recovery of susceptible variants in this transposon tagged population seemed to be possible due to a number of reasons. Firstly, the changes were quantitative towards partial susceptibility. Race specific resistance *Cf* genes in tomato showed also a semidominant phenotype if screened in a quantitative manner (Hammond-Kosack and Jones, 1994). Secondly, chromosome 5 in potato is known to contain other resistance and physiological genetic components (Collins *et al.*, 1999; Leonards-Schippers *et al.*,

1994; Oberhagemann *et al.*, 1999). These loci could probably be efficiently mutagenised due to active linked transposition of *Ds* near *R1*. Thirdly, the highly heterozygous nature of potato could permit the identification of dominant mutations.

The *Ds* flanking sequences identified in variants 994 and 1000 showed striking homology to either *cis*-responsive regions or receptor-like protein kinases. The genes involved could play a role in the *R1* defence related HR resistance pathway. So probably, in both variants the *Ds* insertions have caused a mutation in defence related genes causing an altered reaction to *P. infestans*. This result demonstrates the use of a stringent inoculation method for the selection of *P. infestans R1* resistance variants, that have led to the identification of transposon tagged mutants in the *R1* resistance gene mediated HR signal transduction pathway. The two characterised mutants are therefore, candidates for the isolation of a class of genes specifically involved in the *R1* gene mediated resistance pathway in potato.

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Chapter 5

Identification and characterisation of *Solanum tuberosum* protein kinase homologs required for the *Phytophthora infestans R1* resistance reaction

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Abstract

A *Ds* transposon-mutagenised potato population was screened for resistance to *Phytophthora infestans* mediated by the *R1* gene. This resulted in the identification of genes required for the *R1* resistance mediated signalling pathway leading to HR in potato. In one mutant, the DNA sequences flanking two independent *Ds* insertions both showed high homology to the serine/threonine protein kinase domain of the rice *Xanthomonas* resistance gene *Xa21*, including all conserved domains and a conserved intron position. PCR analysis identified a total of 11 different kinase homologs, named *Solanum tuberosum* protein kinase (StPK), in different potato genotypes independent of the *R1* locus. We demonstrate the tagging and partial isolation of two StPK loci, *Rpr-1* and *Rpr-2*, that are probably required for *P. infestans R1* resistance. The biological correlation between the observed *P. infestans R1* resistance mutation and the molecularly identified *Ds* transposon insertions is discussed.

Introduction

The cloning of *R* genes that mediate gene-for-gene type resistance to bacterial, fungal, oomycete, viral, and nematode pathogens has so far identified 5 classes of genes based on common characteristics including nucleotide binding sites, leucine-rich repeats, transmembrane domains and serine/threonine protein kinases (Bent, 1996; Ellis and Jones, 1998; Hammond-Kosack and Jones, 1997). The shared motifs among R proteins suggest conservation in controlling strain-specific pathogen resistance in plants by similar signalling mechanisms. Genetic mapping and sequence analysis showed frequent clustering of *R* genes with different resistance specificities at complex loci (Jia *et al.*, 1997; Ori *et al.*, 1997; Parniske *et al.*, 1997; Song *et al.*, 1997). Despite these insights into R gene structure, much remains to be elucidated about the mechanisms by which *R* proteins recognise pathogens and transduce signals in the plant cell to initiate defence responses.

To date, only a small number of putative *R* gene signal transduction components have been identified (reviewed in Innes, 1998). Barley *rar1* and *rar2* mutations partially suppress resistance conferred by several powdery mildew resistance genes (Jørgensen, 1996; Peterhänsel *et al.*, 1997), *Cladosporium fulvum Cf9* resistance in tomato is weakened by *rcr1* and *rcr2* mutations (Hammond-Kosack *et al.*, 1994) while *Prf* and *Pti* in tomato are required for *Pto* resistance to *Pseudomonas syringae* pv *tomato* (Salmeron *et al.*, 1994; 1996; Zhou *et al.*, 1995; 1997). Mutant screens in Arabidopsis have identified a number of genes involved in plant pathogen interactions, *NDR1* (Century *et al.*, 1995), *EDS1* (Parker *et al.*, 1996), *PAD1*, *PAD2*, *PAD3* and *PAD4* (Glazebrook *et al.*, 1996) and *PBS1*, *PBS2* and *PBS3* (Warren *et al.*, 1999). Most of these mutations affect the function of a subset of *R* genes (Aarts *et al.*, 1998; Warren *et al.*, 1999) or combinations of double mutations significantly decrease *R* gene resistance (Glazebrook *et al.*, 1997; McDowell *et al.*, 2000). This indicates the occurrence of different signalling pathways for resistance reactions that are also partially redundant.

In potato the *R1* gene confers resistance to specific races of the oomycete *Phytophthora infestans* which contain the *AVR1* gene product. Earlier studies identified minor *S. tuberosum* or *S. demissum* genes that influence or even suppress *R* gene expression (El-Kharbotly *et al.*, 1996b; Ordoñez *et al.*, 1997). Backcrossing to different *S. tuberosum* genotypes showed that besides the normal HR response, also partial

resistance or an intermediate HR response of dominant *R* gene mediated resistance reactions from wild *Solanum* species can occur (Graham, 1963; McKee, 1962; Toxopeus, 1958; 1959). So far, 19 QTLs were mapped in *S. tuberosum* populations segregating for partial *P. infestans* resistance including one on chromosome 5 in the region of the *R1* gene (Leonards-Schippers *et al.*, 1994). In this same chromosomal region, also QTLs for maturity and vigour were identified (Collins *et al.*, 1999; Oberhagemann *et al.*, 1999). These QTLs very likely represent minor genes that play a role in both the *R* gene mediated HR resistance responses and developmental pathways that indirectly influence the resistance response. This region on chromosome 5 also contains several resistance genes with specificity to different other pathogens (Jong *et al.*, 1997; Kreike *et al.*, 1994; Ritter *et al.*, 1991; Rouppe van der Voort *et al.*, 1998). These characteristics make this chromosomal region in the potato genome an attractive target for transposon tagging mutagenesis to identify mutants in disease resistance pathways.

To understand the mechanism of R1 protein-mediated recognition and determine components in the signal transduction pathways leading to effective resistance, a transposon tagging strategy was developed to identify mutants with loss of *R1* gene mediated resistance (Enckevort *et al.*, 2000a; Chapter 3). The somatic selection and regeneration of plants from single cells facilitated the production of a large population of clonal regenerants with independent *Ds* insertions. A stringent inoculation method to screen the tagging population for *P. infestans R1* resistance identified four putative transposon tagged mutants with an altered *R1* resistance phenotype (Chapter 4). Of these, mutant 1000 showed the most consistent altered phenotype, which was comparable to the susceptible parental phenotype. A second putative mutant, mutant 487, displayed a minor but clear deviation from the normal *R1* type HR resistance response. Preliminary sequence analysis on the *Ds* insertions in these putative mutants, identified for mutant 1000 significant homology to receptor kinase-like proteins. This demonstrated that the screening of the transposon-mutagenised population is a promising method for the identification of HR signalling mutants with a quantitative effect. The corresponding genes could be useful in further elucidation of the *R1* resistance mediated signalling pathway leading to the *R1* type HR resistance response in potato. Here, we report on further molecular analyses characterising the two *Ds* insertion loci in mutant 1000. Multiple homologs of the

isolated *Solanum tuberosum* protein kinase (StPK) were identified in several potato genotypes, independent from the presence of the *R1* resistance locus.

Materials and methods

Plant material

J91-6167-2, a *Phytophthora infestans R1* resistant diploid potato genotype (El-Kharbotly *et al.*, 1994), was crossed to the susceptible fertile diploid genotype 87-1024-2 (Jacobsen *et al.*, 1989). From the resulting population J92-6400 the *R1* resistant J92-6400-A16 was selected for its high transformation competence (El-Kharbotly *et al.*, 1995) and a population of *Ds* T-DNA containing potato plants was produced (El-Kharbotly *et al.*, 1996a). BET92-Ds-A16-416 (Ds416), having a two copy repeat *Ds* T-DNA insertion on chromosome 5 (Jacobs *et al.*, 1995), was crossed to J89-5040-2 (RUG87-1022-2 X RUG87-1030-9; Jacobsen *et al.*, 1989) to select among others Ds53-34 for linkage between the *R1* gene and the *Ds* T-DNA (El-Kharbotly *et al.*, 1996a). The *Ac* transposon containing primary transformant TM17-2 (Enckevort *et al.*, 2000b; Chapter 2) was crossed with Ds53-34 and *R1* resistant seedlings with active *Ds* transposition due to the presence of the *Ac* transposase source were selected (Enckevort *et al.*, 2000a; Chapter 3). Protoplast regeneration and hygromycin selection resulted in a *Ds* tagging population of almost 2000 *R1* resistant plants (Enckevort *et al.*, 2000a; Chapter 3). Screening for *P. infestans* resistance identified four putative mutants with an incomplete *R1* gene function (Chapter 4). Two of these mutants, 1000 and 487, are further analysed here together with several of the parental plants form the different crossings.

Southern analysis

Plant genomic DNA was isolated from greenhouse-grown leaves (Pereira and Aarts, 1998) and restricted with *Hin*dIII and *Eco*RI to analyse on Southern blots. Probes were derived from cloning vectors of *Ac* using *Bam*HI and *Hin*dIII digestion to obtain a 5' *Ac* probe and *Hin*dIII digestion solely to obtain an internal *Ac* probe. The 5' *Ac* probe was

used in hybridisation to determine the presence and positions of the *Ac* as well as the *Ds* elements in the different genotypes. Since *Ds* is derived from *Ac*, the 5' *Ac* probe is also able to identify the *Ds* element (*Ds* is derived form *Ac* by deleting the internal *Hin*dIII fragment and introducing pNOS promoter and the NPT II kanamycin resistance gene in the *Xba*I site; Pereira *et al.*, 1992). Additional hybridisation of the same blots with an internal *Ac* probe was performed to specifically characterise the presence and position of *Ac*.

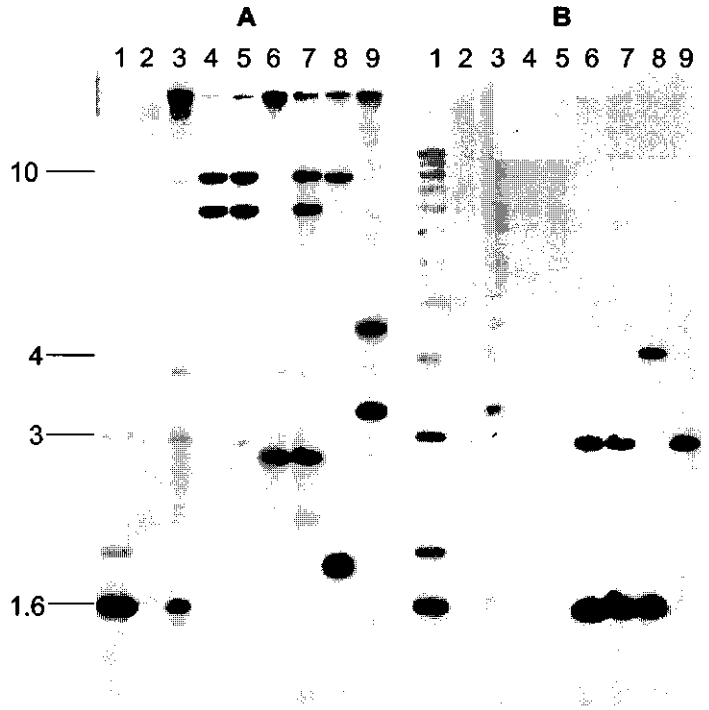


Fig. 1 *Hin*dIII digested genomic DNA hybridised to the 5' Ac probe (**A**) or the internal *Ac* probe (**B**). *Lane 1* shows the 1.6-kb marker hybridisation. The *R1* resistant crossing parent J91-6167-2 (*lane 2a* and *b*) and the susceptible crossing parent 87-1024-2 (*lane 3a* and *b*) contain no *Ac* or *Ds* elements. The primary transformant Ds416 contained two *Ds* T-DNA loci (*lane 4a*), Ds53-34 inherited both *Ds* T-DNA loci (*lane 5a*) as did EE96-4312-28 (*lane 7a*). EE96-4312-28 inherited from TM17-2 (*lane 6a*) the *Ac* element. In mutant 487 (*lane 8a*) and mutant 1000 (*lane 9b*), both regenerated from EE96-4312-28, the *Ds* elements transposed to new positions and *Ac* seems to be missing. In TM17-2 (*lane 6b*) a complete *Ac* (1.6-kb internal *Hin*dIII fragment) and a d*Ac* (2.9 kb) are present. Mutant 487 (*lane 8b*) inherited d*Ac* as a different restriction fragment due to the insertion of *Ac* in d*Ac* (see text). In mutant 1000 (*lane 9b*) *Ac* got lost and only d*Ac* is present

Isolation of Ds and Ac flanking sequences

For analysing the transposon insertion sites, flanking sequences of *Ds* and *Ac* were isolated by Inverse PCR (IPCR; Triglia *et al.*, 1988) as described (Chapter 4). The primer set used identified only 5' flanking sequences for *Ac* and *Ds*. To obtain additional and longer 5' flanking sequences a second restriction combination was used, in which genomic DNA was restricted with *Msc*I followed by *Hind*III and after ligation linearised by *Cla*I. The obtained fragments were amplified by the previously described IPCR protocol, separated on agarose gel, cloned and sequenced (Chapter 4).

Thermal asymmetric interlaced PCR (Liu and Whittier, 1995) was also used to obtain additional *Ds* and *Ac* transposon flanking sequences. Sets of nested primers designed on the 5'- and the 3' site of the *Ac* transposon (Tsugeki *et al.*, 1996; Ds5-1, 5-2, 5-3, 5-4 and Ds3-1, 3-2, 3-3, 3-4) were combined with 4 different degenerated primers (Liu and Whittier, 1995; AD1 to 4) or the two degenerate primers described by Tsugeki *et al.* (1996; renamed AD5 and 6). The three step PCR reactions were performed according to Tsugeki *et al.* (1996). Primers AD3, AD5 and AD6 with *Ac/Ds* 5' primers and primers AD5 and AD6 with *Ac/Ds* 3' primers yielded for Ds53-34, TM17-2 and mutant 1000 specific products which were cloned and sequenced like the IPCR products.

Results

Southern blot analysis

The mutants, 487 and 1000, were characterised molecularly to examine the causal relationship between the *Ds* insertion sites and the observed phenotype. In the different genotypes, DNA blot analysis (Fig. 1) identified the *Ds* and *Ac* insertions with specific *Hind*III fragments (Fig. 2). New positions of the two *Ds* elements confirmed that both mutants were derived from different *Ds* transposition events in EE96-4312-28 during protoplast regeneration (Fig. 1 and Fig. 2).

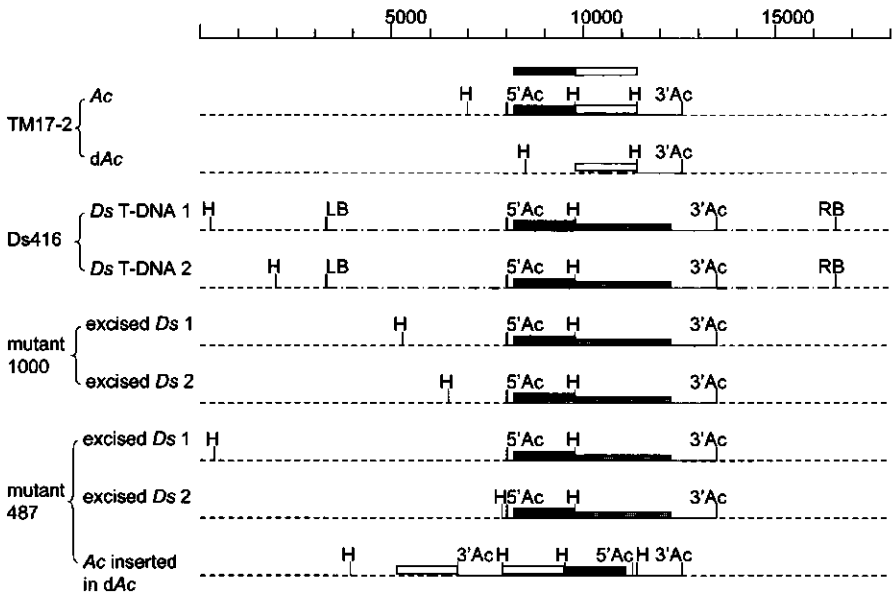


Fig. 2 Genomic positions of *Ac*'s, *Ds* T-DNA's and excised *Ds*'s in TM17-2, Ds416, mutant 1000 and mutant 487 relative to *Hind*III (H) positions deduced from Fig. 1. 5' and 3' *Ac* sequences are marked, *Ds* is derived from *Ac* and contains a nos promoter and the NPTII kanamycin resistance gene (grey bars), the black bar represents the 5' *Ac* probe and the white bar represents the internal *Ac* probe. LB and RB mark the left and right border of the T-DNA's

For the *Ac* parental line TM17-2, previous analysis showed the presence of one functional *Ac* (*Ac*) and one deleted *Ac* (d*Ac*; Enckevort *et al.*, 2000b; Chapter 2). The TM17-2 *Ac* position (Fig. 1, lane 6; Fig. 2) is inherited by EE96-4312-28 (Fig. 1, lane 7), but this fragment was surprisingly not present in the protoplast regenerants mutant 487 and 1000 (Fig. 1, lane 8 and 9). Hybridising the same *Hind*III blot with the internal *Ac* probe (Fig. 1) confirmed the presence of *Ac* (1.6 kb) and d*Ac* (2.9 kb) in TM17-2 (Fig. 1, lane 6; Fig. 2). EE96-4312-28 inherited both *Ac* and d*Ac* (Fig. 1, lane 7) but the mutants 487 and 1000, which are clonal regenerants from EE96-4312-28, missed either *Ac* or d*Ac*. In mutant 487 the d*Ac* structure had changed to a 4-kb *Hind*III fragment (Fig. 1, lane 8). Combining the *Hind*III restriction pattern with IPCR sequence information (see below) revealed that in mutant 487 *Ac* is inserted in the d*Ac*, 48 bp before the *Hind*III site (Fig. 2). So, this mutant was regenerated from a cell in which both *Ac* and *Ds* had transposed to new positions. In mutant 1000 the 1.6-kb *Ac* internal

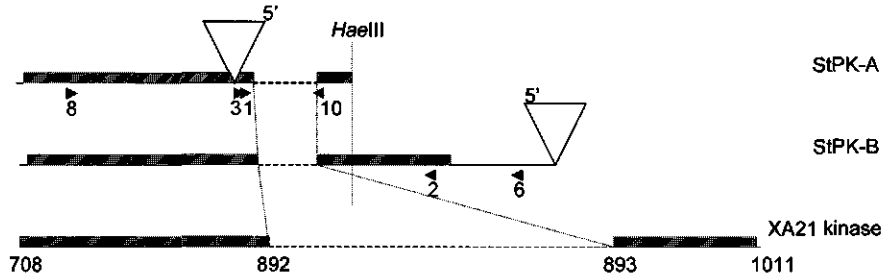
*Hin*dIII fragment was absent (Fig. 1, lane 9) and only the d*Ac* fragment was present. So, also in the cell from which this mutant was regenerated, *Ac* excised during or after *Ds* transposition had occurred. Since no re-insertion of *Ac* was observed, this mutant 1000 is a stable *Ds* mutant in which no reversion is expected.

Identification of Ds and Ac insertion loci

Transposon insertion site flanking sequences for both mutant 487 (100 and 192 bp) and mutant 1000 (288 and 496 bp) were obtained by IPCR and reported earlier (Chapter 4). Additional *Ds* and *Ac* flanking sequences were subsequently obtained by IPCR and TAIL-PCR and characterised further. As expected, from Ds53-34 the *Ds* flanking sequences from the *Ds* T-DNA construct pHPT::*Ds*-Kan (Pereira *et al.*, 1992) were isolated. For TM17-2 the recovery of different *Ac* flanking sequences indicated active somatic *Ac* transposition to independent new insertion sites. In mutant 487 the flanking sequences obtained by IPCR and combined with the Southern blot analysis (see above) identified a new insertion of *Ac,* 48 bp adjacent to the *Hin*dIII site in d*Ac* (Fig. 2). No additional *Ds* flanking sequences were obtained.

In mutant 1000, for one *Ds* insertion the 3' *Ds* flanking sequence was isolated using TAIL-PCR (540 bp; Fig. 3). Combining the 5' 288-bp and the 3' 540-bp flanking sequence for this *Ds* insertion revealed the expected 8-bp target site duplication (Hehl and Baker, 1990). The sequence flanking this *Ds* insertion showed at protein level 50% identity to XA21 (Song *et al.*, 1995). For the second *Ds* insertions the IPCR and TAIL-PCR only extended the 5' flanking sequence from 496 to 1309 bp (Fig. 3). This sequence covered a complete serine/threonine protein kinase ORF with 44% identity to the serine/threonine protein kinase domain of XA21 including the conserved intron position (Fig. 3). All eleven protein kinase specific domains with conserved features were present (Fig. 3) as well as all the 14 conserved amino acids (Hanks *et al.*, 1988). Domain VI (consensus DLKPEN) and domain VIII (consensus G(T/S)XX(Y/F)XAPE) are indicative of serine/threonine specificity (Hanks *et al.*, 1988). The two *Ds* insertion loci displayed 84% identity at the protein level while in the intron region they showed only 52% nucleotide identity. This was a clear indication that the isolated *Ds* flanking *Solanum tuberosum* protein kinase (StPK) represented two distinct *Ds* insertion loci in mutant 1000, StPK-A and StPK-B.

A



B

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Pto             ----------------------------MGSKYSKATNSINDALSSSYLVPFESYRVPLVD  33
Ptil            MSCFSCCDDDDMHRATDNGPFMAHNSAGNNGGQRATESAQRETQTVNIQPIAVPSIAVDE  60
StPK-B          ------------------------------------------------IKTHQLVSYHE  11
StPK-A          ------------------------------------------------IKTNQLISYHE  11
XA21 kinase     ------------------------------------------------------------

                              * *I       *       II*                *III
Pto             LEEATNNFDH-KFLIGHGVFGKVYKGVLRDGAKVALKRRTPESSQGIEEFETEIETLSFC  92
Ptil            LKDITDNFGS-KALIGEGSYGRVYHGVLKSGRAAAAIKKLD-SSKQPDREFLAQVSMVSRL 118
StPK-B          IQQATNNFDDKSNLIGEGSSGSVYKGILSIGTVVAIKVLDLENEQVCKRFDTECKVMRNV  71
StPK-A          IQQVTNNFDG-SNLIGEGSSGSVYKGTLSSGTTVAIKVLDLENEQVCKRFXTECEVMRNV  70
XA21 kinase     ---------FAPTNLLGSGSFGSVYKGKLNIQDHVAVKVLKLENPKALKSFTAECEALRNM  52

                    IV                          V
Pto             RHPHLVSLIGFCDERNEM-----ILIYKYMENGNLKRHLYG-----SDLPTMSMSWEQRL 142
Ptil            KDENVVELLGYCVDGGFR-----VLAYEYAPNGSLHDILHGRKGVKGAQPGPVLSWAQRV 173
StPK-B          RHRNLVPVITTCSSDYIR-----GFVMPIMPNGSLENWLYKED--------RHLNLHQRV 118
StPK-A          RHRNLVPVITTCSSDYXX-----AFVLKYMSXGSHENWLYREV--------RHLNLLQRV 117
XA21 kinase     RHRNLVKIVTICSSIDNRGNDFKAIVYDFMPNGSLEDWIHPETN--DQADQRHLNLHRRV 110

                                  VI*     *            ***VII
Pto             EICIGAARGLHYLHTRA---IIHRDVKSINILLDENFVPKITDFGISKKGTELDQTHLST 199
Ptil            KIAVGAAKGLEYLHEKAQPHIIHRDIKSSNILLFDDDVAKIADFDLSNQAPDMAARLHST 233
StPK-B          TVMLDAAMAVEYLHHCHVAPIVHCDLKPANVLLDEDMVAHVGDFGISKILAISKSMAYTE 178
StPK-A          TVMLDAAMAIEYLHHGNDTVIVHCDINPANVLLDEDMVAHVGDFGISKILAASKSLTQTE 177
XA21 kinase     TILLDVACALDYLHRHGPEPVVHCDIKSSNVLLDSDMVAHVGDFGLARILVDGTSLIQQS 170

                     VIII     *           *  IX*
Pto             V----VKGTLGYIDPEYFIKGRLTEKSDVYSFGVVLFEVLCARSAIVQSLPREMVNLAEW 255
Ptil            R----VLGTFGYHAPEYAMTGQLSSKSDVYSFGVVLLELLTGRKPVDHTLPRGQQSLVTW 289
StPK-B          T-----LGTLGYIAPEYGSEGIVSASGDVYSYGIMLMEVLTKRRPTDEDICNENLDLRKW 233
StPK-A          T-----LGTLGYIAPEYGSEGIVSASGDVYSYGIMLMEVLTKRR---------------- 216
XA21 kinase     TSSMGFIGTIGYAAPEYGVGLIASTHGDIYSYGILVLEIVTGKRPTDSTFR-PDLGLRQY 229

                X                                                   XI   *
Pto             AVESHNNGQLEQIVDPNLADKIR--------------PESLRKFGDTAVKCLALSSEDRP 301
Ptil            ATPRLSEDKVKQCVDARLNTDYP--------------PKAIAKMAAVAALCVQYEADFRP 335
StPK-B          ITQSFS-GSMMDVVDANLFSEEEQITCKS--------EMCIASMIELALDCTKKMPESRV 284
StPK-A          ------------------------------------------------------------
XA21 kinase     VELGLH-GRVTDVVDTKLILDSENWLNSTNNSPCRRITECIVWLLRLGLSCSQELPSSRT 288


Pto             SMGDVLWKLEYALRLQESVI 321
Ptil            NMSIVVKALQPLLPRPVPS- 354
StPK-B          TMKEVVKRLNKIK------- 297
StPK-A          --------------------
XA21 kinase     PTGDIIDEL---------- 297
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Fig. 3 A) Schematic representation of the isolated *Ds* flanking sequences from mutant 1000 (StPK-A and StPK-B) and their alignment to the serine/threonine protein kinase domain of XA21 (aa 708- 1011). The large triangles represent the *Ds* positions, the intron region is a dashed line and the small black triangles represent primer positions of EE1 (1; 5'-aca ttg ggc act ctt gga tac a), EE2 (2; 5'-tct tga ttc tgg cat ttt ctt tg), EE3 (3; 5'-cct gac aca aac cga gac att), EE6 (6; 5'-aac aat gcc ttt ctt ctc), EE8 (8; 5'-gca cat tat caa gtg gaa cta cg) and EE10 (10; 5'-ctg agc cgt act ctt aaa aga acg). **B)** Amino acid alignment of Pto, Pti1, StPK-B, StPK-A and XA21. The eleven conserved domains of a protein kinase are numbered and the conserved amino acids are marked (*). Bold domains are specific for serine/threonine recognition. The N-glycosylation site is underlined

Identification of Solanum tuberosum protein kinase (StPK) homologs

Several sets of primers were designed and used in PCR for the identification of StPK homologs in *R1* resistant and susceptible plants. Primers EE1 and EE2 (Fig. 3) identified in the *R1* resistant parental clone J91-6167-2, the susceptible crossing parent 87-1024-2 and several *R1* resistant and susceptible progeny plants (J92-6400-A1, -A2, -A3, -A4, -A5 and -A6), a product of expected size of about 470 bp and a second product of about 370 bp (except for A1). Sequencing the PCR products derived from J92-6167-2, 87-1024-2, J92-6400-A1 and -A4 identified on basis of homology 10 different StPKs (Table 1). StPK-A was not identified in any of the plants by using this primer combination. From the susceptible parental clone 87-1024-2, StPK-B was isolated. Two additional StPK homologs, StPK-C and -D were identified several times in both *R1* resistant and susceptible clones. StPK-D was the 370-bp PCR product and had a deletion of 108 bp making it very likely a non-functional StPK. From the *R1* resistant plants two additional StPK homologs, StPK-E and -I were isolated and from the susceptible plants 5 additional homologs were isolated, StPK-F, -G, -H, -J and -K (Table 1). The isolation of so many StPK homologs is an indication that these serine/threonine protein kinases represent a multigene family in *S. tuberosum.* This was confirmed by DNA hybridisation, since StPK-B identified over 30 hybridising fragments in *Hin*dIII or *Eco*RI digested genomic DNA of resistant or susceptible potato genotypes (data not shown).

Table 1 Homologs of *Solanum tuberosum* protein kinases (StPK) isolated from *R1* resistant and susceptible clones using primers EE1 and EE2 (Fig. 3)

StPK homolog	% identity to StPK-B	R1r parent J91-6167-2	rr parent 87-1024-2	R1r progeny J92-6400-A4	rr progeny J92-6400-A1	total clones
StPK-B	100		1			1
StPK-C	92	2		3	2	7
StPK-D Δ	82	5	3	5		13
StPK-E	91			1		1
StPK-F	91		1			1
StPK-G	86		1			1
StPK-H	90				1	1
StPK-I	89	1				1
StPK-J	91		1			1
StPK-K	94				2	2
total		8	7	9	5	29

Δ deletion locus

A second set of primers, EE3 and EE6 (Fig. 3), of which primer EE6 is located downstream of the second exon of StPK-B, was specific enough to identify StPK-B in all analysed plants. So, this StPK homolog is present in 87-1024-2 (identified with EE1 and EE2), in J92-6167-2 and in several tested *R1* resistant and susceptible progeny of population J92-6700, including -A16 from which the tagging population was derived (data not shown).

A third set of primers, EE8 and EE10 (Fig. 3), was designed on the low homologous regions between StPK-A and StPK-B and identified specifically the StPK-A locus after *Bgl*II digestion. Analyses of all the parental genotypes used in the different crossings to obtain the parental seedling EE96-4312-28, showed that StPK-A is inherited from the susceptible parent 87-1024-2 through 3 backcrossings into EE96-4312-28 (data not shown). In mutant 1000 this StPK locus is tagged in the first exon by one of the two *Ds* transposons.

Discussion

Mutant 1000 was selected on basis of an altered *P. infestans R1* resistance phenotype. The discovery that two *Ds* transposons were inserted in genes homologous to a part of the serine/threonine protein kinase domain of the rice *Xa21*

resistance gene made this mutant a subject for further studies. Clonal sibling mutant 487, regenerated from the same parental seedling as mutant 1000, showed only a minor deviation from the *R1* type HR resistance response. The transposon insertion flanking sequences in mutant 487 showed no significant homology in the public databases (Chapter 4). DNA blot analyses of both mutants confirmed here the presence of two independent *Ds* insertions at new positions in the potato genome. Surprisingly, in mutant 1000 *Ac* got lost, making selection of revertants impossible but provided a stable mutant. Additionally, in mutant 487 *Ac* transposed to a new position in the deleted *Ac* (d*Ac*) sequence present.

IPCR facilitated the isolation of additional flanking sequences for the *Ds* insertion sites. For mutant 487 this identified the *Ac* insertion in d*Ac* but no additional flanking sequences were obtained which could have been more informative for the *Ds* insertions. The *R1* type HR resistance response in mutant 487 was slightly altered from the parental phenotype (Chapter 4). While in the parental seedling 75% of the inoculated leaves showed a clear *R1* type HR response, for mutant 487 this was reduced to 41% of the inoculated leaves with 43% of the inoculated leaves showing necrotic regions covering 5 to 20% of the leaf and little sporulation. The altered phenotype observed might be due to somatic variation that could occur during protoplast regeneration (Ramulu *et al.*, 1989). Furthermore, the phenotype of mutant 487 (for the *R1* resistance reaction and transposon position) was clearly different from mutant 1000, while they were both regenerated from the same parental seedling EE96-4312-28. This is an additional (indirect) indication for the unique phenotype and genotype observed for mutant 1000.

Mutant 1000 showed a much more susceptible phenotype, compared to parent EE96-4312-28 and mutant 487. After leaf inoculation with *P. infestans* race 0, colonisation and sporulation were observed in 50% of the inoculated leaves. A lower amount of necrosis, indicating absence of the *R1* type HR response, gave this mutant a susceptible phenotype similar to the susceptible parent TM17-2. Only 16% of the inoculated leaves showed the normal *R1* type HR resistance response, so the mutation in regenerant 1000 seemed to have an intermediate effect on the *R1* type HR resistance reaction (Chapter 4). Flanking sequences of the two identified *Ds* insertions in mutant 1000 displayed similarity to serine/threonine protein kinases with highest homology to the serine/threonine protein kinase domain of the *Xanthomonas* resistance gene *Xa21* from rice (Song *et al.*, 1995). Although the protein identity was

less than 50%, all characteristic protein kinase domains and conserved amino acids were present in the potato insertion loci StPK-A and StPK-B (except for domain X and XI in StPK-A), including the putative intron position at exactly the same position in the serine/threonine specific domain VIII. Therefore, it is probable that these serine/ threonine protein kinases are similarly functional in the signal transduction pathway leading to *P. infestans* resistance and perhaps other pathogens.

Surprisingly, the StPKs are more homologous to the protein kinase domain of the rice resistance gene *Xa21* than to the earlier identified Solanaceous tomato resistance genes *Pto* (Martin *et al.*, 1993) and *Pti* (Zhou *et al.*, 1995). More surprisingly, since a homolog of *Pto* was mapped to the *R1* chromosomal V area (Leister *et al.*, 1996). These tomato serine/threonine protein kinases are functional in the signal transduction pathway leading to a hypersensitive response reaction upon infection with *Pseudomonas syringae* pv. *tomato* strains expressing the avirulence gene *avrPto* (Zhou *et al.*, 1997). In *Xa21*, other rice homologs (Tarchini *et al.*, 2000) and in the StPK-A and StPK-B the conserved intron position in domain VIII indicates a conserved gene family among monocots and dicots. No intron position was identified in the tomato serine/threonine kinases *Pto* and *Pti1*. Among the 11 kinase specific domains only minor differences were observed between the potato kinases and *Xa21* on one hand and *Pto* and *Pti1* on the other hand (Fig. 3). But overall amino acid homology determined that the potato sequences were more related to XA21 than to the tomato kinases Pto and Pti1. Domain IV, with no general consensus, showed a high homology between XA21 and the potato sequences while Pto and Pti1 contained different amino acids in this area. Whether this difference determines a clear difference in function or signalling pathway for these kinases needs to be studied.

Primers designed on either StPK-A or StPK-B were used in PCR on several *R1* resistant and susceptible plants. In total 9 additional homologs were identified which probably belong to a large family of serine/threonine protein kinases in potato. DNA hybridisation revealed over 30 hybridising fragments in a single potato genotype (data not shown), indicating also the multigene family character of these kinases in potato. Primer EE6, designed on the non-coding sequences downstream of the second exon, identified specifically StPK-B that was present in all plants analysed, independent of the presence of the *R1* gene. Primers EE8 and EE10 combined with *Bgl*II restriction analysis, identified that StPK-A is present in the susceptible parent 87-1024-2 that was used to produce the starting population from which J92-6400-A16 was selected (El-

Kharbotly *et al.*, 1995). After transformation of the *Ds* T-DNA and 3 backcrosses, including selection for recombination between the *R1* gene and the *Ds* T-DNA, StPK-A was still present in the parental seedling EE96-4312-28. Therefore, it is very likely that this allele is present on chromosome 5, linked to *R1* and tagged by *Ds* in mutant 1000.

As shown, both *Ds* transposon insertions in mutant 1000 are loci that occur solely or also in plants that do not carry the *R1* gene. Therefore, it is very unlikely that StPK-A or StPK-B are the *R1* gene itself. The *Ds* mutagenised StPK loci were designated respectively *rpr-1* and *rpr-2* (required for *Phytophthora infestans* resistance). Both homologs cover a complete (or almost complete) serine/threonine protein kinase ORF with all conserved characteristics including a conserved intron position. The *Ds* insertion in *Rpr1* and near *Rpr2* probably influence their expression explaining the incomplete *R1* type HR resistance reaction in mutant 1000. A mutation in one or two genes of a multigene family (Gilliland *et al.*, 1998) or a specific structure due to transposon or T-DNA insertions or inversions (Bender and Fink, 1995; English and Jones, 1998; Nam *et al.*, 1999; Stam *et al.*, 1998) are examples of a sufficient detectable alteration in the phenotype giving a dominant effect due to a recessive mutation.

If the StPK homologs are similar to the *Xa21* gene structure with an LRR additional to the kinase domain, then in StPK-A the *Ds* insertion, 46 bp upstream of the intron, would probably form a truncated LRR protein without a functional kinase domain. This putative truncated LRR domain could possibly compete with the functional LRR-kinase genes, reducing or delaying the signal transduction to exhibit partial *P. infestans* resistance.

StPK-B contains a *Ds* insertion downstream of the serine/threonine protein kinase domain. For this insertion *Ds* 5' promoter activity (Rudenko *et al.*, 1994) could result in the production of an antisense RNA. Post transciptional gene silencing due to the formed aberrant RNA could result in a reduction of kinase activity making the signalling pathway leading to the *R1* type HR response less effective. This might explain the semi-dominant mutation leading to a mutated *R1* resistance phenotype in regenerant 1000. A delay in HR response makes escape of the *P. infestans* from necrotic regions possible, resulting in sporulation and further colonisation of the infected leaves. The evaluation of which hypothesis explains the partially increased susceptibility in the mutant awaits functional complementation and genetic segregation of the mutated alleles.

Chapter 6

General discussion

Transposon tagging strategies for highly heterozygous diploid potato

The aim of the research described in this thesis was the development of a transposon mutagenesis strategy in diploid potato *(Solanum tuberosum)* for the isolation of genes of which the product is unknown. The autonomous maize transposable element *Ac* was shown to function in potato after introduction through *Agrobacterium transformation* (Knapp *et al.*, 1988; Pereira *et al.*, 1991). For a targeted two-component tagging strategy the non-autonomous *Ds* element was introduced in a specifically selected potato genotype (El-Kharbotly *et al.*, 1995). As transposons are known to transpose preferentially to nearby positions in the genome (Jacobs *et al.*, 1995; Jones *et al.*, 1990; Lawrence *et al.*, 1993; Osborne *et al.*, 1991), efficient targeted tagging of closely linked genes is possible. Therefore, over 60 individual transformed clones were identified with mapped *Ds* T-DNA positions covering all 12 chromosomes of the genetic map of potato (El-Kharbotly *et al.*, 1996a). Individual transformants were then selected with *Ds* linked to the gene of interest for the development of a directed strategy to isolate tagged mutants of the specific gene. In this research the genetic dissection of resistance to the oomycete *Phytophthora infestans*, mediated by the *R1* gene, was chosen to develop the system. The *R1* gene was mapped on chromosome 5 of potato (Leonards-Schippers *et al.*, 1992), in a region with many loci known to confer resistance to different pathogens as well as QTLs for *P. infestans* resistance and potato plant maturity and vigour. These characteristics make this chromosomal region in potato an interesting target for transposon mutagenesis and gene identification.

Development of Ac and Ds tagging lines in diploid potato

To develop transposon tagging strategies, population development by selfings and the use of homozygous lines is one of the basic requirements. After the induction of transposition events, crossing to a genotype recessive for the gene of interest enables the production of a large progeny population for the identification of a tagged mutant. Important in this perspective are the frequencies of independent transpositions obtained in parental lines and the frequencies of germinal transmission of transposition events in progenies. For developing tagging strategies in the tetraploid cultivated

potato, *Solanum tuberosum* L. (2n = 4x = 48), the availability of dihaploids (diploids) made analysis more amenable. However, diploid potato is usually self-incompatible preventing the production of selfed progenies. Therefore, several generations of crossing and selection were needed to obtain vigorous plants, homozygous for the gene of interest.

Chapter 2 of this thesis (Enckevort *et al.*, 2000b) describes the results obtained for *Ac* transposition frequencies in primary transformants and for *Ac* and *Ds* transposition frequencies from progeny analysis in potato. The cell autonomous *GBSS* gene was shown to be an accurate visual excision assay to study *Ac* excision frequencies in primary transformants of potato (Pereira *et al.*, 1991). High levels of early excision in tubers were observed indicating that directly after transformation the expression levels of the *Ac* transposase was sufficient to induce *Ac* excision and subsequent re-insertion in the primary transformed cell. Some primary transformants showed late *Ac* transposition in single cells or small sectors resulting in many independent transposition events in one single plant. The observed *Ac* excision frequency in the analysed transformants turned out to be independent of copy number, suggesting that the *Ac* T-DNA positions in the potato genome determined the transposition phenotype. This strongly supported the position hypothesis proposed by Peterson for transposition frequencies in maize (Peterson, 1977). Furthermore, in one of the transformants rapid proliferation of *Ac* copies was detected supporting that amplification of *Ac* is associated with transposition (Yoder, 1990). This can result in rapid increase of maize *Ac* transposable elements in potato like in other heterologous plants.

In sexual progeny of *Ac*-active lines several seedlings were detected with new independent *Ac* re-insertions indicating the germinal transmission of independent transposition events. The occurrence of these new *Ac* insertions showed that the production of *Ac* insertion lines in potato through seed is a potential method to produce transposon insertion mutants. Somatic excision events also still occurred in the seedlings indicating that *Ac* expression continued in the progeny. Crossing *Ac* clones, which had shown early *Ac* transposition, with *Ds* clones showed that somatic *Ds* transposition occurred in all analysed *Ac-Ds* seedlings. These seedlings appeared to be highly chimaeric with high levels of late independent *Ds* transpositions.

From the analysis first on *Ac* and later on *Ds* excision frequencies in different lines we observed that in each *Ac* line early and continuous transposition occurred until

Ac inserted in a rather stable position in the genome. The autonomous *Ac* controlled by its own promoter produces high transposase expression levels confirming early excision when inserted in some positions in the genome. After inserting in other genome positions, transposase activity can drop to a lower level and transposition of *Ac* or *Ds* occurs only in a few cells. This also implies that in a potato plant with a relatively stable *Ac* position producing lesser transposase, a *Ds* transposon insertion can result in a stable mutant phenotype from which the tagged gene can be isolated.

Production of large tagging populations

1) Germinal transmission of transposition

The populations produced to study germinal transmission of *Ac* transposition were derived from *P. infestans R1* resistant diploid potato clones. Therefore, from the *Ac* progeny plants with somatic *Ac* transposition described in Chapter 2 (Enckevort *et al.*, 2000b), plants could be selected for the presence of the *P. infestans R1* resistance gene. These *R1* resistant *Ac* containing plants were crossed to *R1* resistant *Ds* containing plants for the selection of *R1* homozygotes with presence of *Ac* and *Ds* (R1Ds/R1-;Ac/-). Since several generations of crossing and selection were needed, it took four growing seasons before R1Ds/R1-;Ac/- with active transposition were identified (unpublished). These R1Ds/R1-;Ac/- plants were used in crossings to susceptible potato plants (r1r1) and by now thousands of seeds are available in which after selection for the presence of *Ds*, the screening and selection for susceptible *R1* mutant phenotypes can be performed.

2) Production and selection of somatic transposition events

While the production of R1Ds/R1-;Ac/- potato plants turned out to be very time-consuming, meanwhile, an alternative method for directed transposon tagging in diploid potato was developed. Potato has proven to be particularly amenable to tissue culture techniques, including rapid methods for micro-propagation and protoplast isolation (Wenzel, 1994). Heterozygous *R1* resistant diploid potato with a linked *Ds*

was crossed to active *Ac* plants and *R1* resistant seedlings with active somatic *Ds* transposition were selected (R1Ds/r1-;Ac/-). These seedlings were highly chimaeric with independent *Ds* excision in about 10% of the cells. Protoplast regeneration and cell specific hygromycin selection for *Ds* excision enabled the direct selection and regeneration of a large population of *R1* plants with independent new *Ds* insertion sites (Chapter 3; Enckevort *et al.*, 2000a). Molecular analysis confirmed that the selected hygromycin resistant regenerants potentially represented 2000 independent *Ds* re-insertions starting from a T-DNA position linked 18 cM to the *R1* locus on chromosome 5 of potato. This tagging population formed the basis for the identification of putative *R1* resistance mutants. Furthermore, this population could be useful in a reverse genetics approach to identify chromosome 5 insertion mutants on basis of the transposon insertion site sequence (Ballinger and Benzer, 1989; Das and Martienssen, 1995; Speulman *et al.*, 1999; Wisman *et al.*, 1998; Zwaal *et al.*, 1993). All available *Ds* insertion mutants can be used in future studies on gene structure and function in potato.

R gene signalling

Mutants in the Phytophthora infestans R1 resistance pathway

The R1Ds/r1-;Ac/- tagging population was derived through protoplast regeneration and hygromycin selection for *Ds* transposition, enabling the recovery of independent *Ds* transpositions in the regenerated plants. These plants were screened using a detached leaf assay for *Phytophthora* inoculation yielding 33 putative resistance variants in the first screen. They usually showed a minor deviation from the normal *R1* type HR resistance response and no clear susceptible phenotype was found. In a second year putative variants were re-tested and their *R1* type HR resistance reaction was more intensively compared to the phenotype of the parental seedling they were derived from. Quantification of the altered resistance response enabled the identification of four putative mutants with significantly higher levels of susceptibility for *P. infestans* race 0 when compared to the parental seedlings (Chapter 4). However, in all mutants the *R1* type HR response was observed upon inoculation but, very often the HR reaction was weak and unable to restrict the growth of *P. infestans* hyphae in

this plant. The resulting colonisation of the leaf enabled the formation of spores, identifying mutants as susceptible to *P. infestans* race 0 infection when compared to the phenotype of the parental seedlings. Probably the population size was too limited for isolating the *R1* tagged mutant. The stringent inoculation method for the selection of *P. infestans R1* resistance variants very likely only identified mutants in the *R1* resistance gene mediated HR signal transduction pathway. These mutants are candidates for the isolation of genes specifically involved in the *R1* gene resistance pathway in potato.

R gene signalling pathways

Besides the cloning of *R* genes that mediate gene-for-gene type resistance reactions, also a number of putative *R* gene signal transduction components have been identified by mutation (reviewed in Innes, 1998). Although the identified mutations reduce resistance conferred by specific *R* genes, most do not completely eliminate all defence responses. In *rar1* and *rar2* plants, mutants for the barley powdery mildew mediated *Mla12* resistance (Torp and Jørgensen, 1986), an intermediate level of susceptibility to powdery mildews was demonstrated (Freialdenhoven *et al.*, 1994; Jørgensen, 1996). Screens for loss of resistance in tomato identified *rcr1* and *rcr2*, both mutations weaken *Cladosporium fulvum Cf-9* resistance but neither mutation allows sporulation of the fungus (Hammond-Kosack *et al.*, 1994; Hammond-Kosack and Jones, 1994). *Rcr3* is required specifically for the *C. fulvum Cf-2* mediated resistance reaction (Dixon *et al.*, 2000). *Prf* and *Pti* are required for *Pto* resistance to *Pseudomonas* syringae pv *tomato* (Salmeron *et al.*, 1994; 1996; Zhou *et al.*, 1995; 1997). The tomato *rcr1*, *rcr2*, *rcr3*, *prf* and *pti* mutants are reported to affect pathogen resistance conferred by single *R* genes, but these mutations have not yet been comprehensively analysed for their effect on other *R* gene mediated resistance pathways.

Identification of Arabidopsis mutants in resistance gene reactions, *ndr1* (Century *et al.*, 1995), *eds1* (Parker *et al.*, 1996), *pad1*, *pad2*, *pad3* and *pad4* (Glazebrook *et al.*, 1996), and *pbs1*, *pbs2* and *pbs3* (Warren *et al.*, 1999), revealed downstream genes acting in HR signalling pathways and shared signal transduction components. Most of these mutations affect the function of a subset of *R* genes tested. Plants carrying the *ndr1* mutation allow extensive growth of several previously avirulent races of *P.*

syringae pv *tomato*, however, these plants can still produce a visible hypersensitive response to some of these bacterial strains (Century *et al.*, 1995). Additionally, *ndr1* plants are only partially susceptible to most isolates of *Peronospora parasitica* (Century *et al.*, 1995; 1997).

The *ndr1* and *eds1* mutants are affected in resistance against both bacterial and oomycete pathogens. Interestingly, *R* genes that are strongly suppressed by one of these Arabidopsis mutations are not greatly affected by the other mutation, indicating that *NDR1* and *EDS1* may be critical for different signalling pathways (Aarts *et al.*, 1998; McDowell *et al.*, 2000). All combinations of double mutations between *pad1*, *pad2* and *pad3* displayed significant decreases in resistance to *P. parasitica*. These data indicate that these three *PAD* genes may be functioning in different pathways that are partially redundant for resistance to *P. parasitica*. Mutant *pad4* appeared to completely suppress resistance mediated by the *P. parasitica R* genes *RPP2* and *RPP4* (Glazebrook *et al.*, 1997; Innes, 1998). The mutants *pbs1*, *pbs2* and *pbs3* are affected in the expression of *RPS5* and several other *R* genes that confer resistance to various strains of *P. syringae* pv *tomato* and *P. parasitica* (Warren *et al.*, 1999).

The *Ds* flanking sequences in the mutants 994 and 1000 showed striking homology to the *cis*-responsive regions of defence related genes and to receptor-like protein kinases respectively. Both mutants might be involved in the signalling pathway leading to a specific HR response that is probably shared by different recognition components besides the *R1* gene analysed here. These potential signal transduction pathway genes become increasingly interesting for understanding and manipulating resistance defence reactions in plants (Melchers and Stuiver, 2000). Identification of these genes is of interest since they are likely to be less dependent on a specific *R-Avr* gene recognition and therefore, probably more durable in maintaining a resistance phenotype.

Semi-dominant mutations due to transposon insertions

The transposon insertion analysis of mutant 1000 identified two independent *Ds* insertions in two DNA sequences with homology to serine/threonine protein kinases which very likely play a role in the signal transduction pathway of the *R1* mediated HR resistance response (chapter 5). The *Ds* mutation in one or both of these *Solanum*

tuberosum protein kinases (StPK) can confer the altered *P. infestans* resistance phenotype of mutant 1000. At present we are unable to explain the precise mechanism by which the transposon mutation of one or both kinase genes can cause the *P. infestans* susceptible phenotype, but here we suggest several possibilities that can be addressed by experimental verifications.

There are several reports showing that a mutation in one or two genes of a multigene family is sufficient to result in a detectable alteration of the phenotype. For example, mutation of one of the eight active *Arabidopsis* actin genes can be deleterious to the plant (Gilliland *et al.*, 1998). Several *rat* mutations in Arabidopsis showed a dominant or semi-dominant phenotype when crossed to wild-type plants indicating a possible gene dosage effect (Nam *et al.*, 1999). The *Ds* insertion in the first exon of the StPK-A, giving *rpr1,* could actually be responsible for the *R1* type HR response mutation in mutant 1000. Despite the fact that it is very likely that some StPK homologs in this mutant are still functional, a reduced expression level of one StPK with additive effect could already result in a reduced dosage in expression level and activity of these kinases. Moreover, if *Rpr1* is similar to the *Xa21* gene structure with an LRR additional to the kinase domain, then the *Ds* insertion in the kinase domain could result in a truncated LRR protein lacking a functional kinase domain. A truncated LRR domain could still compete with other functional LRR-kinase genes for an elicitor. Examples of a truncated gene with a dominant effect are the *Cl-1* mutant allele from maize (Paz-Ares *et al.*, 1990) and the *m5-2* mutant of *Arabidopsis* (Glover *et al.*, 1998). The effect might be a reduction or delay in the signal transduction resulting in a delayed HR response visible as a partial *P. infestans* resistance reaction.

At the *rpr2* locus in mutant 1000 the *Ds* is inserted into the untranslated 3' region of StPK-B. The effect of this mutation might influence the stability or localisation of the mRNA transcript. In the well characterised dominant Arabidopsis mutant *rat5* two T-DNA copies are integrated as a tandem repeat in the 3' untranslated region of a histone H2A gene (Mysore *et al.*, 2000). In this case the structure and function of the RAT5 protein is not mutated but the stability and localisation of the mRNA is changed to contribute tot the dominant mutation effect.

Moreover, the *Ds* insertion in *rpr2* might also result in the formation of aberrant RNA transcript due to 5' *Ds* promoter activity (Rudenko *et al.*, 1994). The formation of double-stranded RNA triggers silencing (Mette *et al.*, 1999; Vaucheret *et al.*, 1998;

Waterhouse *et al.*, 1998), that could subsequently reduce the expression of the *Rpr* gene and of StPK homologs resulting in reduction or delay in the signal transduction.

The specific structure combined with an inverted orientation of two *Ds* transposon insertions in two homologous genes (possibly both members of a complex StPK locus) could also contribute to a dominant effect. From literature examples are known in which dominant mutations occur due to specific structures of transposon insertions and a number of findings suggest that inverted repeats can be particular potent silencers of gene expression (Selker, 1999). In tobacco a strong correlation was observed between a particular structure, comprised of two directly repeated *Ac* elements flanking a *SPT* marker gene and silencing of *SPT* marker genes (English and Jones, 1998). Transposable elements have also been postulated to be involved with silencing interactions between *nivea* alleles in *Antirrhinum majus*. In this case not the presence of the transposable element itself, but structural features like inversions of the *nivea* allele and multiple copies resulted in a dominant effect due to a recessive mutation (Bollmann *et al.*, 1991). In the *PAI* gene family of *Arabidopsis*, specifying the enzyme phosphoribosylanthranilate isomerase, the presence of an inverted repeat of 2 *PAI* genes was correlated with methylation and gene-silencing (Bender and Fink, 1995). In petunia post transcriptional gene silencing was found associated with both an inverted repeat arrangement of the transgenic DNA and DNA methylation (Stam *et al.*, 1998). An inverted repeat structure is probably present in mutant 1000 if the *Ds* inserts are in adjacent *Rpr* genes of a complex StPK cluster.

Final conclusion

The development of an efficient transposon mutagenesis strategy for the isolation of genes in diploid potato resulted in a somatically generated population of almost 2000 independent *Ds* insertional potato mutants. This population, saturated for chromosome 5 insertions near the *R1* locus, can be useful in a reverse genetics approach using chromosome 5 gene specific primers combined with transposon primers. Such `target selected gene inactivation' approaches have been developed in a number of eukaryotic transposon systems (Ballinger and Benzer, 1989; Zwaal *et al.*, 1993) including maize (Das and Martienssen, 1995) and Arabidopsis (Speulman *et al.*, 1999;

Wisman *et al.*, 1998). In potato the identification of *Ds* mutated chromosome 5 genes in a reverse genetics strategy could provide new ways for functional gene analysis.

The identification of a *Ds* transposon tagged *R1* mutant was not successful in the developed somatically generated tagging population. In 1998 we also succeeded in the selection of R1R1 potato clones with a linked *Ds* and active transposition to use for the selection of germinal transmitted transposition events. Crossings in 1999 to a susceptible potato genotype were performed and thousands of seeds are now available for a new screen for the identification of *R1* mutants. This might result in the identification of a *R1* tagged mutant for the molecular isolation of the *R1* gene. Knowing the structure and protein of the *R1* gene is still of great importance, since this gene is the main player in activating the *R1* type HR resistance response in potato upon infection with *P. infestans* race 0.

From the mutant screen in the somatically generated tagging population four putative *R1* mutants were identified. In two of these mutants, mutant 994 and mutant 1000, a correlation between the *Ds* insertion sequence and the observed phenotype was made. The isolated StPKs from mutant 1000 will be useful in further studies to elucidate the HR signalling pathways in potato. In mutant 994 the *Ds* mutation near the *cis*-response elements with homology to the tomato defence related TAPG2 and TAP1 genes need to be studied further in more detail. These insertion loci might be related to the minor genes that are located in the *R1* gene cluster of chromosome 5. Knowing and understanding this locus in the *Solanum* genome will be crucial in understanding *P. infestans* resistance levels in potato. This increased knowledge might contribute to the development of durable *Phytophthora infestans* resistant potato cultivars in the 21st century.

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The late blight disease, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is a serious threat to the potato crop every growing season. This has, for example, led to the disastrous Irish famine in the middle of the 19th century, and continued in the 20th century to remain a serious problem for potato growers. Since the early 1980s *P. infestans* populations changed more rapidly and epidemics even increased in their severity. Resistance breeding stimulated the introduction of resistance genes (*R* genes) from wild *Solanum* species into cultivated potato, *Solanum tuberosum*, but newly occurring virulent races of *P. infestans* circumvented these *R* gene mediated resistances and no cultivars with durable resistance were obtained. At the moment, methods using fungicides supervised by spraying control via decision support systems are the only available control measures.

Characteristic for *R* gene type mediated resistance reactions is the hypersensitive response (HR) leading to local cell death causing necrotic spots at the site of attempted infection. Genetic analysis of HR mediated resistances showed that activation of HR is highly specific and induced upon recognition between a specific *R* gene in the plant and a corresponding avirulence gene (*Avr* gene) in the pathogen. Insights in the molecular mechanisms underlying this HR resistance reaction in *Solanum* species might facilitate the development of potato cultivars that are more durable in maintaining a resistance phenotype.

A two component *Ac-Ds* transposon tagging strategy in diploidised potato was developed to identify and isolate genes involved in the *R1* gene mediated resistance response to *P. infestans*. Transposable elements are molecular genetic tools to mutate and identify genes. The transposable elements *Ac* and *Ds* were first characterised in maize and their molecular isolation led to the identification of maize genes that were tagged by these elements. The autonomous *Ac* element is able to transpose by itself and also to induce transposition of the non-autonomous *Ds* element that is transposase defective. Introduction of these elements in heterologous species demonstrated their utility for isolating genes in self-fertilising plant species. Also in the highly heterozygous and tetraploid potato, the *Ac* and *Ds* transposable elements were shown to be functional. A cell autonomous visual marker gene for potato, the granule bound starch synthase gene (*GBSS* gene), enabled a refined characterisation of *Ac* transposition in potato. Further molecular characterisation showed high levels of *Ac-Ds* transposition both somatically and germinally, so that suitable populations could be generated for tagging purposes.

The production of clones homozygous for the gene of interest that are normally required for efficient tagging strategies, turned out to be time consuming in potato due to self-incompatibility at the diploid level. Therefore, an alternative method based on somatic transposition was developed for the direct selection of transposition events instead of recovering germinally transmitted transpositions. Highly chimaeric *Ac-Ds* seedlings with active *Ds* transposition linked to the *R1* resistance gene on chromosome 5 of potato were selected. Protoplasts were isolated from actively transposing seedlings and using the hygromycin excision selection marker, regenerants could be selected with new independent *Ds* insertions. The resulting *R1* resistant transposon mutagenised population of almost 2000 hygromycin resistant regenerants formed an ideal start for the identification of an *R1* tagged mutant, or other *Ds* insertional mutants with an altered *R1* resistance response.

The somatically regenerated tagging population was analysed for the *P. infestans R1* type HR resistance response, using a detached leaf assay for *P. infestans* inoculation. In a primary screening, 33 potential *R1* resistance variants showing partial susceptibility to *P. infestans* race 0 were identified. These results were further quantified using stringent inoculation conditions on replicate samples leading to the identification of four putative mutants with a distinctly altered *R1* resistance response. In these putative mutants less than 50% of the inoculated leaves showed the *R1* type HR response and clear colonisation with sporulation of *P. infestans* was observed. The flanking sequences of the *Ds* insertion sites in these putative *R1* mutants were analysed and in two cases a potential biological correlation between the insertion sequences and the phenotype was evident. One putative mutant contained a *Ds* insertion in a region with auxin and abscisic acid response cis-elements homologous to a specific region (TAPIR) of the tomato defence related genes *TAPG2* and *TAP1*.

The second *P. infestans R1* resistance mutant, mutant 1000 with a striking susceptible phenotype was characterised in more detail. Two *Ds* insertions were identified and the insertion site flanking sequences both showed high homology to serine/threonine protein kinases. The *Ds* insertion sites turned out to be homologous but not identical, indicating two independent *Ds* insertions in homologous but not identical genes. Both sequences showed protein identity to all the conserved regions of serine/threonine protein kinases and they contained a conserved intron position. The closest homology was to the serine/threonine protein kinase domain of the

Xanthomonas resistance gene *Xa21*, which is involved in the induction of a HR resistance response in rice. This indicates that the isolated *Solanum tuberosum* protein kinase (StPK) homologs are candidate genes involved in resistance gene activity in potato. Further specific molecular analyses identified at least 11 homologs by sequence, which probably belong to a large family of serine/threonine protein kinases in potato. Both homologs in which the *Ds* transposons are inserted were present in susceptible parental potato clones. Therefore, it is unlikely that the isolated sequences represent the *R1* gene itself. The mutated StPKs were designated *rpr1* and *rpr2*, required for *Phytophthora infestans* resistance gene 1 and 2. Studying these mutants and the StPKs involved might help in understanding the pathway leading to HR resistance in potato.

Samenvatting

De aardappel is na maïs, rijst en tarwe mondiaal gezien het vierde voedselgewas, terwijl in Nederland aardappel het belangrijkste voedselgewas is met een totale productie van 8.200.000 ton in 1999. De grootste bedreiging voor een aardappelteelt tijdens het groeiseizoen vormt de aardappelziekte. De veroorzaker van deze ziekte, *Phytophthora infestans*, werd voor het eerst aangetroffen in Europa in 1845. In dat jaar verspreidde zich een epidemie over West-Europa met grote gevolgen voor de aardappelopbrengsten in België, Nederland en Frankrijk. In 1846 had een *Phytophthora* epidemie in Ierland een zodanig dramatisch effect op de totale aardappelteelt dat hongersnood, ziekte en emigratie volgden.

Voorheen werd *P. infestans* aangeduid als een schimmelziekte maar tegenwoordig wordt dit pathogeen, een oomyceet niet meer ingedeeld in het rijk van de Fungi, maar in het rijk Protista, verwant aan de algen. Een *Phytophthora* infectie kan zich zeer snel ontwikkelen, met name als de weersomstandigheden gunstig zijn (vochtig en warm). Een enkele infectiebron, zowel in een veld als op bijvoorbeeld een afvalhoop van aardappelen, kan tot een epidemie leiden als er niet voldoende maatregelen worden getroffen. Sinds 1980 komt naast het A1 paringstype ook het A2 paringstype in Nederland en Europa voor. Naast de altijd al aanwezige ongeslachtelijke voortplanting kan er nu ook een geslachtelijke voortplanting van *P. infestans* optreden. Dit heeft tot nieuwe en agressievere isolaten geleidt en de oösporen (geslachtelijke sporen) die kunnen overleven in de grond vormen in een nieuw groeiseizoen een infectiebron voor een nieuwe epidemie. Een *Phytophthora* epidemie heeft grote gevolgen voor het aardappelgewas, de opbrengst en de kwaliteit van de aardappel bij bewaring. Momenteel is het toepassen van fungiciden, eventueel gecombineerd met beslissings-ondersteunende waarschuwingssystemen, de enige wijze waarmee een *Phytophthora* epidemie bestreden kan worden. Een grote infectie in een aardappelperceel maakt afdoding van het loof noodzakelijk en daarmee een vroegtijdige beëindiging van de teelt.

Veredeling met als doel de introductie van resistentiegenen uit wilde aardappelsoorten in de gecultiveerde aardappel, *Solanum tuberosum*, heeft slechts in beperkte mate resultaten opgeleverd. De eerste introductie van resistentiegenen uit de wilde aardappel *S. demissum* leken in de eerste helft van de 20^{e} eeuw effectief te zijn, maar al snel bleek dat *Phytophthora* zich kon aanpassen en dat de nieuwe resistenties werden omzeild. Deze resistenties, die gebaseerd zijn op een enkel resistentiegen (*R* gen) waren niet bestand tegen de snel veranderende *Phytophthora* populatie en

derhalve niet duurzaam. Sindsdien is de veredeling zich gaan concentreren op niet *R* gen specifieke resistenties, ook wel horizontale resistentie genoemd. Een probleem is echter dat hierbij vaak toch *R* genen betrokken zijn en dat de genetische basis van horizontale resistentie onbekend is.

Karakteristiek voor *R* gen resistenties is de hypersensitieve respons (HR). Als een *Phytophthora* spore kiemt en een epidermiscel penetreert wordt deze herkend door de plant en als reactie treedt necrose op. Een aantal cellen rond de infectieplaats sterft af en *Phytophthora* kan zich niet verder ontwikkelen. Genetische analyse van HR resistentie mechanismen laat zien dat een specifieke genetische factor in de plant (het *R* gen) en een specifieke genetische factor in het pathogeen (het *Avr* gen) noodzakelijk zijn voor de herkenning en daarmee de werking van resistentie. Als het pathogeen een specifiek *Avr* gen mist dan herkent de plant het pathogeen niet en treedt infectie op. Meer inzicht in de moleculaire mechanismen die ten grondslag liggen aan de herkenning tussen een *R* gen en een *Avr* gen zal een bijdrage leveren aan onze kennis over de werking van resistenties tegen pathogenen in planten. Deze kennis kan bijdragen aan de ontwikkeling van aardappelrassen met duurzame resistentie tegen *P. infestans*.

Het onderzoek dat beschreven is in dit proefschrift had als eerste doel de moleculaire isolatie van het *P. infestans R1* resistentie gen uit aardappel. *R1* is één van de *R* genen die in het begin van de 20^{e} eeuw geïntroduceerd zijn in aardappelrassen maar niet duurzaam zijn gebleken. Uit eerdere studies aangaande de genetische achtergrond van *R1* resistentie is gebleken dat één genetische factor, gelegen op aardappel chromosoom 5, bepaalt of een specifiek isolaat van *Phytophthora* herkend wordt door een *R1* resistente aardappelplant of niet. Aangezien het verder onbekend is hoe de resistentie gereguleerd wordt was het noodzakelijk om het *R1* gen moleculair te isoleren voor verder onderzoek. Er is gekozen voor een transposon tagging strategie die het mogelijk maakt om op basis van de selectie voor *R1* mutanten het gen te isoleren dat *R1* resistentie bepaalt.

Transposons zijn stukjes DNA die zich in een cel kunnen bewegen. Het transposase enzym dat door een transposon gemaakt wordt, herkend specifieke DNA fragmenten aan de uiteinden van een transposon. Vervolgens knipt dit enzym zichzelf, of een ander transposon met dezelfde uiteinden, uit een DNA streng (excissie). Het transposon verplaatst zich vervolgens naar een andere plaats in het DNA (insertie). Het hele proces van excissie en insertie wordt transpositie genoemd. Als een

transposon insertie optreedt in een bestaand functioneel gen dan kan het gevolg zijn dat dit specifieke gen niet meer functioneert. Een gen dat eerst verantwoordelijk is voor bijvoorbeeld *Phytophthora* resistentie kan die functie dan niet meer vervullen en de plant wordt vatbaar. De plaats van de transposon insertie markeert een stuk DNA en het gen, dat door de insertie niet meer functioneert, is 'getagged'. De transposon tag kan vervolgens gebruikt worden om met behulp van moleculaire technieken het gen te isoleren voor verdere analyse en karakterisatie.

Voor de ontwikkeling van een transposon tagging strategie in aardappel is gekozen voor een twee-componenten strategie. Naast het gebruik van het oorspronkelijk uit maïs afkomstige autonome *Ac* transposon, dat zelf transpositie kan ondergaan, is een tweede transposon, *Ds*, gebruikt. Het *Ds* transposon bezit wel de karakteristieke uiteinden van een *Ac* transposon maar is voor transpositie afhankelijk van het transposase enzym van een *Ac* transposon. Eerder onderzoek had al aangetoond dat het *Ac* transposon na introductie in aardappel, via *Agrobacterium* transformatie, functioneert (springt). In hoofdstuk 2 van dit proefschrift is het transpositie gedrag van *Ac* in aardappel nader bestudeerd. Het *Ac* transposon is in een aardappelplant gebracht samen met een excissie marker. Als excissie marker is gebruik gemaakt van het korrelgebonden zetmeelsynthase gen (*KGZ* gen) dat in aardappel zorgt voor de productie van amylose zetmeel. Door een plant te gebruiken die van zichzelf geen amylose produceert, kan na transformatie de expressie van het geïntroduceerde *KGZ* gen met daarin het *Ac* transposon bestudeerd worden. *KGZ* met *Ac* is niet functioneel en produceert geen amylose; na excissie van *Ac* wordt *KGZ* wel functioneel en wordt er amylose geproduceerd. Deze amylose productie in zetmeel kan zichtbaar worden gemaakt door kleuring met jodium. Zetmeel zonder amylose kleurt rood, zetmeel met amylose kleurt blauw (zie Fig. 2, hoofdstuk 2). Het bleek heel goed mogelijk om in knollen van amylose-vrije aardappelplanten *KGZ* expressie en daarmee *Ac* excissie zichtbaar te maken. Zo is een beeld verkregen van het *Ac* transpositie gedrag in aardappel en zijn geschikte *Ac* aardappelplanten geselecteerd. Verschillende aardappelplanten met actieve *Ac* transposons zijn gebruikt in kruisingen om ook in nakomelingen het *Ac* transpositie gedrag te bestuderen. Daarnaast zijn er ook kruisingen gemaakt met *Ds* bevattende aardappelplanten. Zowel *Ac* als *Ds* blijven actief als transposon in deze nakomelingen. De belangrijkste conclusie was dan ook dat *Ac* en *Ds* in aardappel te gebruiken zijn voor de verdere ontwikkeling van een tagging populatie voor de isolatie van het *R1* resistentiegen.

De volgende stap in de ontwikkeling van een efficiënte tagging populatie was de selectie van planten die: 1) homozygoot zijn voor het *R1* gen (R1R1); 2) een gekoppeld *Ds* transposon bevatten (R1Ds/R1) en 3) actieve *Ds* transpositie vertonen als gevolg van de aanwezigheid van een *Ac* transposon als transposase bron (R1Ds/R1;Ac/-). Bij aanvang van het onderzoek in 1995 waren er R1Ds/r1- aardappelplanten aanwezig. Een probleem bij aardappel is zelf-incompatibiliteit waardoor het niet mogelijk is om zelfbestuivingen te maken. Daarom zijn er kruisingen gemaakt tussen R1Ds/r1- en R1-/r1-; Ac/- planten en uit de nakomelingpopulaties zijn R1Ds/R1-;Ac/- planten geselecteerd. Kruising met r1-/r1- planten heeft veel zaden opgeleverd en een klein deel van deze is al geanalyseerd om met zekerheid vast te stellen dat de geselecteerde ouderplanten daadwerkelijk R1Ds/R1-;Ac/- zijn. In de overige zaden kan nu gezocht worden naar mutanten die een *Ds* transposon insertie in het *R1* gen bevatten en als gevolg daarvan het *R1* fenotype niet meer bezitten.

Omdat er voor de selectie van R1Ds/R1-;Ac/- planten meerdere seizoenen van kruisen en selecteren nodig waren, is ondertussen een alternatieve transposon tagging strategie ontwikkeld om *R1* mutanten te selecteren (hoofdstuk 3). Uit kruisingen tussen R1Ds/r1- en r1-/r1;Ac/- planten zijn 22 R1Ds/r1-;Ac/- nakomelingen geselecteerd en hun *Ds* transpositie gedrag is intensief bestudeerd. In alle planten met een *Ds* en een *Ac* transposon is *Ds* transpositie aangetoond, meestal in ongeveer 10% van de geanalyseerde cellen. Er is slechts één nakomeling gevonden waar al in een heel vroeg stadium van embryo ontwikkeling *Ds* transpositie was opgetreden. *In vitro* zijn de cellen met *Ds* transpositie geselecteerd door gebruik te maken van een excissie marker, het hygromycine resistentie gen. Een methode om cellen van een plant van elkaar te scheiden is protoplastenisolatie. Vervolgens kan uit elke individuele cel (protoplast) een nieuwe aardappelplant groeien. Door aan het medium, waarin de protoplast zich tot plant ontwikkelt, hygromycine toe te voegen zullen alleen die protoplasten, waarin het *Ds* transposon is gesprongen, uitgroeien tot plant. Er zijn hygromycine resistente protoplast regeneranten (HRPR) verkregen van 16 van de 22 geselecteerde R1Ds/r1-;Ac/- planten. Moleculaire analyse aan deze HRPR planten liet zien dat op een somatische wijze planten waren geselecteerd met elk een onafhankelijke nieuwe *Ds* positie. De 2000 *Ds* mutanten vormden samen de eerste aardappel transposon tagging populatie die geanalyseerd kon worden voor de selectie van *R1* mutanten.

Hoofdstuk 4 van dit proefschrift beschrijft de selectie van mogelijke *R1* mutanten uit de populatie van 2000 HRPRs. Na inoculatie van de HRPR planten met een specifiek *P. infestans* isolaat (fysio 0) tonen de meeste HRPR planten het verwachte *R1* resistentie fenotype; het *R1* gen herkent fysio 0 en er treedt een resistentie reactie op in de vorm van een hypersensitieve respons (HR). Dit is zichtbaar als kleine necrotische puntjes op een blad dat geïnoculeerd is. Bij 33 HRPR planten werd echter een afwijking gevonden in het *R1* reactie fenotype. De necrotische puntjes werden vlekken, *Phytophthora* ontwikkelde zich in het blad en er werden sporen gevormd (zie Fig. 1, hoofdstuk 4). Dit was een indicatie voor het uitblijven van een goede *R1* resistentie reactie en dus een mogelijke mutatie van het *R1* gen in deze planten. In een tweede jaar zijn deze mogelijke *R1* mutanten opnieuw geanalyseerd voor hun fenotype na inoculatie met *Phytophthora* fysio 0. Ditmaal zijn naast 10 tot 25 *in vitro* vermeerderde planten per mogelijke mutant ook de R1Ds/r1-;Ac/- ouderplanten waaruit de HRPR mutanten geregenereerd waren opnieuw geïnoculeerd en de resistentie reactie of het gedeeltelijk uitblijven hiervan is grondig bestudeerd. Voor een groot deel van de HRPR mutanten die geselecteerd waren in het eerste jaar, is niet een duidelijke afwijking van het *R1* resistentie fenotype gevonden. Weliswaar trad er af en toe kolonisatie van *Phytophthora* in het blad op, maar dit werd ook gevonden in de ouders. Er bleek bij nauwkeurige analyse variatie te zijn in het *R1* resistentie patroon van de verschillende R1Ds/r-;Ac- ouders. Een kwantitatieve analyse was vervolgens noodzakelijk en voor 4 mutanten werd een duidelijke afwijking gevonden vergeleken met de resistentie reactie zoals die in de ouder optreedt.

Naast deze fenotypische analyse is voor verschillende HRPR mutanten ook geprobeerd de insertie plaatsen van de *Ds* transposons te isoleren. Met behulp van moleculaire technieken zijn stukjes genomisch DNA, flankerend aan het *Ds* transposon, geïsoleerd. Vervolgens zijn deze stukjes DNA in een databank vergeleken met het DNA van genen waarvan de DNA-structuur en de functie al bekend zijn. Dit kunnen genen uit aardappel zijn, maar ook genen uit andere planten, bacteriën, schimmels of zelfs dieren. Op basis van overeenkomsten tussen stukjes onbekend aardappel DNA en DNA van genen kan een mogelijke functie van het aardappel DNA worden voorspeld. Voor meerdere mutanten bleek het *Ds* transposon geïnserteerd te zijn in een bekend gen. Voor twee mutanten kon direct een mogelijke relatie worden vastgesteld tussen de *Ds* insertie plaats en het gevonden afwijkende *R1* fenotype.

Voor de mutant met als volgnummer 1000 werd homologie gevonden met een eiwitkinase. Hiervan is eerder in rijst en tomaat aangetoond dat deze een functie hebben bij het tot stand komen van een HR reactie na infectie met een pathogeen. Deze opvallende gelijkenis met eiwitkinasen, maakte het zeer waarschijnlijk dat in mutant 1000 de *Ds* transposon insertie via een mutatie zorgt voor het niet functioneren van de *R1* resistentie. Bij een tweede mutant, mutant 994, was het opvallend dat jonge bladeren nog geen goede *R1* HR resistentie vertoonden en oudere bladeren wel. In deze mutant is het *Ds* transposon geïnserteerd vlak bij auxine en abscisinezuur respons-elementen die onder andere eerder gevonden zijn in de tomaten genen polygalacturonase 2 (*TAPG2*) en anionic peroxidase 1 (*TAP1*). Response-elementen spelen een rol bij het induceren van de expressie van genen. Mogelijk zorgt de transposon insertie in mutant 994 er voor dat de genen betrokken bij deze respons-elementen niet of in mindere mate tot expressie komen. Als deze genen een rol spelen bij de *R1* resistentie zal een veranderde expressie kunnen leiden tot een verminderde *R1* resistentie. In deze planten lijkt dat het geval te zijn in jonge bladeren, terwijl in oudere bladeren wel een goede *R1* reactie optreedt. Nader onderzoek aan deze genen zal in de toekomst mogelijk meer informatie geven over de totstandkoming van *R1* resistentie.

Mutant 1000 vertoonde het meest overtuigend een vatbaar fenotype ondanks de aanwezigheid van het *R1* gen. Hoofdstuk 5 beschrijft een nader gedetailleerde analyse van deze mutant. Er zijn twee verschillende *Ds* transposon insertie plaatsen uit deze mutant geïsoleerd die beide homoloog zijn met een eiwitkinase. Er kon aangetoond worden dat het om twee homologen en niet twee identieke eiwitkinasen gaat. Deze kinasen zijn StPK-A en StPK-B, *Solanum tuberosum* protein kinase A en B, genoemd. Daarnaast zijn er in aardappel nog meer van dit soort kinasen aanwezig, waarvan er nu 11 verschillende geïsoleerd zijn. Deze StPKs zijn in aardappel onafhankelijk van het *R1* gen aanwezig. De conclusie is dat de geïsoleerde StPKs niet het *R1* gen zelf zijn, maar dat het om andere genen gaat die wel betrokken zijn bij het tot stand komen van de *R1* resistentie reactie. We noemen daarom StPK-A en StPK-B voorlopig *Rpr1* en *Rpr2*, 'required for *Phytophthora infestans* resistance' genen 1 en 2.

Het onderzoek beschreven in dit proefschrift laat zien dat het mogelijk is om in diploïde aardappel via somatische selectie *Ds* transposon mutanten te selecteren. De screening op *Phytophthora R1* mutanten heeft niet geleid tot de tagging en isolatie van het *R1* resistentie gen. Wél is door de selectie van mutant 1000 een eiwitkinase

geïdentificeerd dat zeer waarschijnlijk betrokken is bij de totstandkoming van een goede *R1* HR resistentie reactie in aardappel. Verder onderzoek met de verkregen zaadpopulatie maakt het mogelijk een mutant te selecteren die tot de isolatie van het *R1* gen zelf leidt. Daarnaast zal onderzoek aan het geïsoleerde eiwitkinase gen tot meer kennis over de *R1* resistentie in aardappel leiden. Mogelijk resulteert dit in een bijdrage tot de ontwikkeling van duurzame resistentie tegen *Phytophthora infestans*.

Nu mijn proefschrift bijna klaar is om gedrukt te worden wil ik hier nog even stilstaan bij de afgelopen jaren en de mensen die voor mij een belangrijke rol hebben gespeeld bij de totstandkoming.

Allereerst mijn promotor Evert Jacobsen. Nadat je me in 1993 teleur moest stellen voor een AIO vacature gaf je me toen wel het vertrouwen om door te gaan met mijn ingeslagen weg. In 1995 maakte je mij dan ook bijzonder gelukkig door me aan te stellen als AIO bij plantenveredeling op een aardappel - *Phytophthora* project. Bij de start van mijn onderzoek was je zeer betrokken en dichtbij om een goede richting te geven aan de opzet. Je hebt me vanaf het begin duidelijk gemaakt dat het eerste doel promoveren was, en niet het isoleren van het *R1* gen. In de loop van de jaren werd ons contact iets minder intensief, mede door je drukke werkzaamheden buiten de vakgroep. Toch stond je altijd klaar om mij te adviseren en te motiveren. Het laatste jaar heb je een zeer belangrijke rol gespeeld in de totstandkoming van mijn eerste artikelen en uiteindelijk het proefschrift. Ik heb het altijd zeer knap van je gevonden hoe snel je om kon schakelen van beleids- en bestuurszaken naar onderzoek en wetenschap. Daarnaast wist je op persoonlijk vlak als dat nodig was ook je inbreng te geven. Evert bedankt.

Hoewel officieel aangesteld bij plantenveredeling heeft mijn bureau altijd op CPRO-DLO gestaan. De samenwerking tussen de afdeling moleculaire biologie van CPRO-DLO en de vakgroep plantenveredeling vormden de basis voor mijn AIO aanstelling. Tijdens de sollicitatie gesprekken had ik er nog geen idee van maar snel werd duidelijk dat ik onder de bezielende leiding van Andy Pereira de wereld van transposon tagging zou gaan ontdekken. Andy, jij als dagelijks begeleider hebt een zeer belangrijke rol gespeeld in mijn ontwikkeling als onderzoeker. Ik heb regelmatig moeite gehad met de vrijheid en ruimte die je me gaf voor de invulling van mijn onderzoek. Nu ben ik je daar zeer dankbaar voor. Ik heb zelf, ondanks veel twijfelen, altijd achter alle keuzes gestaan en ben me thuis gaan voelen in transposon tagging. Jouw kennis, bezieling en relativering hebben er voor gezorgd dat ik vier jaar lang met plezier bij je gewerkt heb. Ik blijf nog in je cluster, maar voor nu: zeer bedankt.

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betekent voor een boer. Helaas heb je door een fatale hersentumor niet meer mogen meemaken dat ik in Wageningen begon aan mijn *Phytophthora* promotieonderzoek. In mijn hart ben je er nog altijd.

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Ellen

Curriculum vitae

Leonora Johanna Gertruda van Enckevort, roepnaam Ellen, werd geboren op 29 augustus 1967 in America, gemeente Horst (L). Na het doorlopen van de lagere school in America volgde zij de eerste vier leerjaren VWO op het Peelland College te Deurne. In 1983 verhuisde zij samen met haar ouders en zussen naar Zuidelijk Flevoland, later de gemeente Zeewolde. Het VWO diploma behaalde zij in 1985 op de Rijksscholengemeenschap te Amersfoort en in datzelfde jaar begon zij de studie plantenveredeling aan de toenmalige Landbouwhogeschool in Wageningen. Tijdens deze studie werd een stageperiode doorgebracht bij Prof. C. H. Bomman op de afdeling Research and Development van het suikerbietenveredelingsbedrijf Hilleshög AB in Landskrona, Zweden. De afstudeervakken betroffen fytopathologie (dr. L. C. Davidse), erfelijkheidsleer (dr. J. H. de Jong) en plantenveredeling (dr. A. Kuipers). Na haar afstuderen aan de Landbouwuniversiteit Wageningen (LUW) in augustus 1991 heeft zij van april tot oktober 1992 als medewerker informatievoorziening gewerkt bij de expositie Plantenveredeling en Biotechnologie op de Internationale Tuinbouwtentoonstelling Floriade in Den Haag - Zoetermeer. Hierna heeft zij gekozen om terug te keren in het onderzoek en bij de vakgroep Nematologie van de LUW werd zij vanaf maart 1993 in de gelegenheid gesteld om door middel van een na-doctoraal onderzoeksproject ervaring op te doen in de moleculaire genetica. Per 1 juni 1995 werd zij aangesteld op een door de Associatie van Biotechnologisch Onderzoeksscholen in Nederland (ABON) gefinancierd AIO project bij de vakgroep plantenveredeling van de LUW en de afdeling Moleculaire Biologie van CPRO-DLO. De resultaten verkregen in dit onderzoek hebben geleid tot dit proefschrift getiteld 'Identification of potato genes involved in *Phytophthora infestans* resistance by transposon mutagenesis'. Sinds 1 augustus 2000 is ze aangesteld als wetenschappelijk onderzoeker moleculaire biologie/ genetica bij de business unit Genomica van Plant Research International B.V., Wageningen Universiteit en Researchcentrum.

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