# Propositions

1. The similar constitution of the *Rpi-blb1* gene cluster and the highly homologous sequences in the primitive diploid *S. bulbocastanum* of series *Bulbocastana* and the advanced polyploid *S. stoloniferum* of series *Longipedicellata* strongly suggests the first being one of the ancestors of the second (This thesis).

2. The presence of several resistance genes against late blight and root-knot nematode in *S. stoloniferum* indicates that extensive screening of *S. stoloniferum* may identify material showing resistance to both diseases (This thesis).

3. Obtaining an accurate depiction of the evolutionary history of all living organisms has been and remains one of biology's great challenges (Rokas and Carroll, 2006. PLoS Biology 4: 1899-1904).

4. The durable management of potato late blight seems more complicated than was expected (Andrivon et al., 2005. Plant Pathology 54: 723-732).

5. Being a PhD is the process to explore myself, others and the world. A positive attitude can turn a "Mission impossible" into a "Mission possible".

6. China is a must-visit country for researchers working on diversity.

7. Wageningen is a small "village" with international researchers from many countries, enabling people to access the amazing variety of cultures throughout the world without even stepping out of Wageningen.

Propositions associated with the PhD thesis of Miqia Wang

**Diversity and evolution of resistance genes in tuber-bearing *Solanum* species**

Wageningen, June 12th, 2007

# Diversity and evolution of resistance genes in tuber-bearing *Solanum* species

**Miqia Wang**

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# Diversity and evolution of resistance genes in tuber-bearing *Solanum* species

Miqia Wang

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op gezag van de rector magnificus
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# Contents

# Chapter 1
# General introduction

## Potato

The potato (*Solanum tuberosum* L. ssp. *tuberosum*) originates from the Andes in South America. Taken by the Spanish to Europe in the 16th century, potato spreads from Europe across the globe (Hawkes 1990). The potato is the world's fourth food crop - after maize, wheat and rice, with a total production of more than 323 million tons in 2005, contributing greatly to the world's food supply, especially to that of developing countries like China. For the top ten producing countries (Table 1), which account for two-thirds of the global potato output, the total value of the crop was estimated at some $40 billion (http://www.fao.org/ag/magazine/0611sp1.htm). The potato is used for human consumption (cooked, baked, processed into French fries or crisps (chips)) or for industrial purposes (dried products and starch production). Bradshaw et al. (2006) summarize that other new uses can be anticipated for the future such as designer starches (Davies 1998) and biopharmaceuticals (Sonnewald et al. 2003).

**Table 1** Top ten potato producing countries, 2005 (Data source: FAO/ESS) (http://www.fao.org/es/ess/top/commodity.html)

| Potato Production | MT |
|---|---|
| 1. China | 73036500 |
| 2. Russian Federation | 36400000 |
| 3. India | 25000000 |
| 4. Ukraine | 19480000 |
| 5. USA | 19111030 |
| 6. Germany | 11157500 |
| 7. Poland | 11009390 |
| 8. Belarus | 8185000 |
| 9. Netherlands | 6835985 |
| 10. France | 6347000 |

## Potato systematics

The tuber-bearing species of the genus *Solanum* provide the potato crop with a secondary gene pool where a broad spectrum of pathogen resistance (including resistance to late blight, nematodes, bacteria and viruses) has accumulated throughout evolution (Ross 1986; Hawkes 1990). Systematic relationships are important criteria

to select material for breeding. Although many wild potato species display a wide range of morphological characteristics, many other species have a general appearance similar to the cultivated potato (Spooner et al. 1992). Systematics within the tuber-bearing *Solanum* is an area that gave rise to several disputes regarding species boundaries. A factor, strongly contributing to the widely conflicting taxonomic treatments of wild potatoes, is that many potato species can hybridize with each other and produce fertile F1 hybrids. Recent taxonomic overviews were provided by Hawkes (1990), Spooner and Hijmans (2001), Spooner and Salas (2006). Based mainly on morphological data, Hawkes (1990) classified potato into 227 species in 19 series. Later, with the access of molecular markers, potato taxonomists used restricted fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSRs), sequences of 5S ribosomal DNA (rDNA) and external transcribed spacer (ETS) (Hosaka et al. 1984; Kardolus et al. 1998; Bryan et al. 1999; Miller et al. 1999; Volkov et al. 2001; Raker and Spooner 2002; Volkov et al. 2003; Sukhotu et al. 2004) to reconstruct and update the systematic relationships within *Solanum* sect. *Petota*. Spooner and Hijmans (2001) reduced the number of tuber-bearing species to 206. One reason to reduce the species number is that many species recognized by Hawkes (1990) are morphologically similar to each other as mentioned above; even potato taxonomists have great difficulty to differentiate them. Another important reason is that differentiation of some species is poorly supported due to the low bootstrap/jackknife value in analyses of molecular data (Spooner and Hijmans 2001). More recently, Spooner and Salas (2006) reduced the number of species further to 188 wild and one cultivated species for section *Petota*, plus three species in section *Etuberosa*.

The complex status of potato taxonomy poses the question to potato genebanks in the world, which taxonomic classification to choose. The present status is that most genebanks use the names of the species recognized by Hawkes with some updates from more recent literature. The unclear taxonomic situation makes choosing material difficult for potato breeders and researchers. It also makes studies related to species richness complicated (Gaston and Williams 1996). Recently Spooner et al. (2005), using amplified fragment length polymorphism (AFLP) markers, have put forward evidence that potato was domesticated in Peru.

## *Phytophthora infestans* and its center of genetic diversity

*Phytophthora infestans* causes late blight, the devastating potato disease responsible for the Irish famine in the mid 1840s (Birch and Whisson 2001). As a consequence one million people starved and another million immigrated to North America (Bourke 1993). At the present time, late blight is still the most devastating disease in potato. The oomycete is currently controlled by frequent applications of fungicides.

*P. infestans* has been studied extensively. In the pathogen's life cycle, three basic steps are involved: formation of mycelium in the host plant, spread of the infection and formation and dispersal of spores. Zoospores are produced by self-reproduction in the asexual life cycle, while oospores are produced by sexual reproduction. Oospores of *P. infestans* can be produced in all tissues of infected potato crops and wild *Solanaceous* hosts. In the winter the oospores stay alive in the soil and germinate at a later stage (Drenth et al. 1995; Andersson et al. 1998; Turkensteen et al. 2000). The appearance of oospores and the enlargement of genetic variation of the pathogen make the late blight resistance breeding more complex.

The Toluca valley (>2600m above sea level), latitude (19°N), and longitude (99°E) in Mexico is an area which is characterized by almost constant cool temperatures and daily precipitation (Grunwald et al. 2000 and 2002). With average daily temperatures between 5 to 10°C (maximum around 20 to 25°C) and high humidity, the climate is ideal for late blight development (Grunwald et al. 2000 and 2002). The sexually reproducing A2 type of *P. infestans,* was discovered in Mexico, spreading quickly through the USA, Europe, Asia and North Africa in the 1970s and 1980s (Fry et al. 1992). The central highlands of Mexico are considered to be the center of genetic diversity for the potato late blight pathogen (Goodwin et al. 1992; Grunwald et al. 2001; Grunwald et al. 2005).

## Late blight resistance genes and quantitative loci

Over the past century, potato breeders have introduced at least 11 late blight resistance (R) genes from *S. demissum* into the cultivated potato (Gebhardt and Valkonen 2001). New races of the pathogen having virulence genes compatible with the R genes in the cultivars soon overcame this type of resistance. In the past fifteen years, researchers and breeders have shifted their attention to species others than *S. demissum*, with the aim to find more durable resistance against *P. infestans*. In Table 2, a summary is

given of late blight resistance genes and quantitative loci detected in cultivated and wild species until now.

**Table 2** Late blight resistance genes and quantitative loci (QTL)

| Source | Gene or QTL | Chromosome | Reference |
|---|---|---|---|
| *S. berthaultii* | *Rpi-ber* | 10 | Ewing et al. 2000; Rauscher et al. 2006 |
| *S. bulbocastanum* | *RB/Rpi-blb1* | 8 | Naess et al. 2000; Song et al. 2003; van der Vossen et al. 2003 |
| *S. bulbocastanum* | *Rpi-blb2* | 6 | van der Vossen et al. 2004 |
| *S. bulbocastanum* | *Rpi-blb3* | 4 | Park et al. 2005 |
| *S. bulbocastanum* | *R12* | 10 | Sanchez et al. 2000 |
| *S. berthaultii* | *R13* | 7 | Sanchez et al. 2000 |
| *S. demissum* | *R2* | 4 | Li et al. 1998 |
| *S. demissum* | *R3a* | 11 | Huang et al. 2005 |
| *S. demissum* | *R3b* | 11 | Huang et al. 2005 |
| *S. demissum* | *R5* | 11 | Huang et al. 2005 |
| *S. demissum* | *R6* | 11 | El-Kharbotly et al. 1996 |
| *S. demissum* | *R7* | 11 | El-Kharbotly et al. 1996 |
| *S. demissum* | *R8* | 11 | Huang et al. 2005 |
| *S. demissum* | *R9* | 11 | Huang et al. 2005 |
| *S. demissum* | *R10* | 11 | Huang et al. 2005 |
| *S. demissum* | *R11* | 11 | Huang et al. 2005 |
| R-gene differentials of Black | *R10, R11* | 11 | Bradshaw et al. 2005 |
| *S. demissum* | *R1* | 5 | Leonards-Schippers et al. 1992; Ballvora et al. 2002 |
| *S. microdontum* | QTL | 4/10 | Sandbrink et al. 2000 |
| *S. microdontum* | QTL | | Bisognin et al. 2005 |
| *S. mochiquense* | *Rpi-moc1* | 9 | Smilde et al. 2005 |
| *S. paucissectum* | QTL | 10/11/12 | Villamon et al. 2005 |
| *S. phureja* | *Rpi-phu* | 9 | Sliwka et al. 2006 |
| *S. phureja* | QTL | 7/12 | Ghislain et al. 2001 |
| *S. pinnatisectum* | *Rpi1* | 7 | Kuhl et al. 2001 |
| *S. tuberosum/S.demissum* | QTL | 8 | Meyer et al. 1998 |
| *S. tuberosum*/*S.spegazzinii* | QTL | 4/5 | Leonards-Schippers et al. 1992 |
| *S. tuberosum*/*S.spegazzinii* | QTL | 3/5/6/9/11 | Oberhagemann et al. 1999 |
| | *Rpi-abpt* | 4 | Park et al. 2005 |
| | *R2*-like | 4 | Park et al. 2005 |

## Use of wild germplasm for cultivar improvement

Apart from breeding for *Phytophthora* resistance a lot of effort has been put into

breeding for other types of biotic stress resistance. Several species have been used in potato virus resistance breeding, including *S. acaule* (Ritter et al. 1991), *S. chacoense* (Marczewski et al. 2004), *S. demissum* (Marczewski et al. 2001), *S. phureja* (Tommiska et al. 1998), *S. tuberosum* subsp. *andigena* (Ritter et al. 1991; Bendahmane et al. 1997; Sorri et al. 1999; Kasai et al. 2000; Celebi-Toprak et al. 2002). S. *stoloniferum* MPI 61.303/34 from the Max Planck Institute in Koln was introgressed into many potato cultivars as the source of resistance to potato virus Y.

Resistance to potato cyst nematodes has been introgressed from *S. tuberosum* subsp. *andigena* (Pineda et al. 1993; Gebhardt et al. 1993), *S. spegazzinii* (Barone et al. 1990; Caromel et al. 2003; Kreike et al. 1993) and *S. vernei* (Jacobs et al 1996). Breeding clone VTN62-33-3 has been used for introgression breeding (directly or indirectly) in many European cultivars like 'Frisia'. *S. tuberosum* subsp. *andigena* accession CPC 1673 confers resistance not only to potato virus X (Bendahmane et al. 1997), but also to cyst nematode *Globodera pallida* (Rouppe van der Voort et al. 1997b). The accession has been the ancestor for many potato cultivars, for example, 'Aziza'. Nowadays, the resistance is effective against *Globodera rostochiensis* in Britain because Ro1 is still the main pathotype there, but its widespread deployment has encouraged the spread of *G. pallida* (Bradshaw et al. 2005). From the information presented above it is clear that the secondary gene pool of potato is widely used for resistance breeding and that many cultivars will contain introgressed genes.

**Resistance genes: genome organization and evolution**

Plant disease resistance genes encode proteins that detect pathogens and most R genes encode proteins that have a putative amino-terminal signaling domain, a nucleotide-binding (NBS) and a series of carboxy-terminal leucine-rich repeats (LRRs). NBS-LRR proteins have been divided into two major classes: those with an amino-terminal TIR (Toll/interleukin receptor) domain (which are known as TIR-NBS-LRR or TNL proteins) and those that encode an amino-terminal coiled-coiled motif (CC-NBS-LRR or CNL proteins) (Meyers et al. 2005). NBS-LRR proteins are numerous and ancient in origin. There are approximately 150 NBS-LRR-encoding genes in *Arabidopsis thaliana* and over 400 in *Oryza sativa* (Meyer et al. 2003; Monosi et al. 2004). NBS-LRR-encoding genes are frequently clustered in the genome. These clusters are the result of both segmental and tandem duplications (Meyer et al. 2003; Monosi et al. 2004; Richly et al. 2002; Leister 2004).

The rate of evolution of NBS-LRR-encoding genes can be rapid or slow, even within an individual cluster of similar sequences. In lettuce, RGC2 genes exhibit heterogeneous patterns of evolution. Depending on their rate of evolution, R genes have been classified in type I and type II (Kuang et al. 2004). The LRR-encoding regions of Type I RGC2 genes evolve rapidly through frequent sequence exchanges between paralogs. In contrast, Type II RGC2 genes evolve slowly, maintaining obvious allelic/orthologous relationships between clades (Kuang et al. 2004).

**NBS profiling**

NBS profiling is a PCR-based approach that specifically targets R genes and their analogs (RGAs). In NBS profiling, genomic DNA is digested with a restriction enzyme, and an NBS-specific (degenerate) primer is used in a PCR reaction towards an adapter linked to the resulting DNA fragments. The NBS profiling protocol generates a reproducible polymorphic multilocus marker profile on a sequencing gel that is highly enriched for R genes and RGAs (van der Linden et al. 2004). NBS profiling was successfully used in potato with several restriction enzymes, and several primers targeted to different conserved motifs in the NBS. The protocol was similarly successful in other crops (including tomato, barley, and lettuce) without modifications. NBS profiling can thus be used to produce markers tightly linked to R genes and R gene clusters for genomic mapping and to mine for new alleles and new sources of disease resistance in available germplasm. The efficiency of the NBS-profiling method for generating RGA markers for resistance loci was demonstrated in apple (Calenge et al. 2005).

**Scope of this thesis**

The aim of this thesis was to study the diversity and evolution of resistance genes in tuber-bearing *Solanum* species. Two approaches were followed. First plant material was analyzed using NBS profiling (chapters 2 and 3). Second, an in depth study was carried out to analyze the dynamics in two specific *P. infestans* resistance genes (chapters 4 and 5),

Wild tuber-bearing *Solanum* species from South and Central America are known as the major sources of resistance towards *P. infestans* but the systematic relationships within the tuber-bearing *Solanum* species obtained with neutral markers have not

resulted in a priori identification of the most promising species for *P. infestans* resistance. NBS profiling was used to study the systematic relationships in a set of over 100 genebank accessions, comprising a broad diversity of tuber-bearing *Solanum* species. The results obtained with NBS profiling are compared to those with AFLP (chapter 2).

Chapter 3 presents the development in genetic diversity of a large set of potato varieties grown in North-Western Europe during the last 70 - 80 years as measured by NBS profiling. Changes in diversity are related to introgression of resistance genes and in some cases NBS profiling markers linked to these genes are identified. This chapter also addresses the homoplasy issue for NBS profiling.

In Chapter 4 the presence of two late blight resistance genes, *Rpi-blb1* and *Rpi-blb2,* was analyzed in depth in tuber-bearing *Solanum* species using gene specific primers. In addition the dynamics of the cluster harboring *Rpi-blb1* was studied.

In chapter 5, R gene diversity of the two late blight resistance genes *Rpi-blb1* and *Rpi-blb2* was explored in accessions of *S. bulbocastanum* and *S. cardiophyllum*. The implications of the results described in this thesis for the exploitation of *Solanum* resources and resistance breeding are discussed.

# Chapter 2

# The utility of NBS profiling for plant systematics: a first study in tuber-bearing *Solanum* species

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**Abstract**

Potato (*Solanum tuberosum* L.) is a crop with a large secondary genepool, which contains many important traits that can be exploited in breeding programs. Systematic relationships are important criteria for researchers and breeders to select materials. We evaluated a novel molecular technique, nucleotide binding site (NBS) profiling, for its potential in phylogeny reconstruction. NBS profiling produces multiple markers in resistance genes and their analogs (RGAs). In this study we used a set of over 100 genebank accessions, representing 47 tuber-bearing wild and cultivated *Solanum* species plus two outgroups species. Results of NBS profiling were compared to those of amplified fragment length polymorphism (AFLP). Cladistic and phenetic analyses showed that the two had similar resolving power and delivered trees with a similar topology. However, the different statistical tests used to show this were inconclusive. Visual inspection of the trees showed that, especially at the lower level, many accessions grouped together in the same way in both trees; at the higher level, when looking at the more basal nodes, only a few groups were well supported. Again this was similar for both techniques. The observation that higher level groups were poorly supported might be due to the nature of the material and the way the species evolved. The similarity of the NBS and AFLP results indicates that the role of disease resistance in speciation is limited.

## Introduction

The potato (*Solanum tuberosum* L.) is a crop with a large secondary gene pool, which contains important traits that can be exploited in breeding programs. In the last few years the identification and cloning of late blight (*Phytophthora infestans*) resistance genes from wild relatives of the cultivated potato has been the subject of many studies (Ballvora et al. 2002; Song et al. 2003; Huang et al. 2005; van der Vossen et al. 2003 and 2005; Park et al. 2005). Systematic relationships within the group of tuber-bearing *Solanum* species are regarded as important criteria to select interesting material. Based on morphological data, relationships among tuber-bearing *Solanum* species have been studied extensively resulting in a classification of 227 species in 19 series (Hawkes 1990). Spooner and Hijmans (2001) updated this by reducing the number of tuber-bearing species to 206. Recently, Spooner and Salas (2006) reduced the number to 188 wild and one cultivated species for section *Petota*, plus 3 species in section *Etuberosa*. Apparently, the boundaries between some of the species in this group are not very clear.

Relationships within the tuber-bearing *Solanum* species have been studied using different molecular markers resulting in new insights at different levels of potato taxonomy. These studies include Restriction Fragment Length Polymorphism (RFLP) markers of chloroplast DNA (cpDNA) (Hosaka et al. 1984; Sukhotu et al. 2004), RFLPs of the nuclear genome (Debener et al. 1990; Miller et al. 1999), Amplified Fragment Length Polymorphism (Kardolus et al. 1998), Simple Sequence Repeat (SSRs) (Raker and Spooner, 2002), cpDNA SSRs (Bryan et al. 1999), sequence data of 5S ribosomal DNA (rDNA) (Volkov et al. 2001), and external transcribed spacer (ETS) (Volkov et al. 2003).

Recently, a novel molecular technique called Nucleotide Binding Site (NBS) profiling was developed (van der Linden et al. 2004). This technique specifically targets resistance genes and their analogs. Resistance (R) genes containing an NBS are numerous in plants and are distributed over all chromosomes (Meyers et al. 2002 and 2003; Monosi et al. 2004). The technique is based on amplification of DNA fragments starting from the conserved NBS domain towards an adaptor which is ligated to a restriction fragment. Primers based upon several conserved motifs (P loop, the kinase-2 motif, and the GLPL motif) within the NBS domain can be used as a starting point. As a consequence this

technique produces gene targeted markers, while other marker techniques like AFLP produce markers randomly in the genome. NBS profiling generates a reproducible polymorphic multi-locus banding pattern and has already been successfully used to identify and map RGAs in potato, apple and lettuce (van der Linden et al. 2004 and 2005; Calenge et al. 2005; Syed et al. 2006).

In this paper we describe the first use of NBS profiling for phylogeny reconstruction and classification of *Solanum* species. To explore the prospects of NBS profiling for systematic research, previously obtained AFLP data (Kardolus 1998) were used as reference. As NBS profiling targets resistance genes and their analogs, we address the question whether this affects the outcome of the systematic analysis by comparing results from NBS profiling to results obtained from AFLP. The role of disease resistance in the evolution of species is discussed.

## Material and methods

### Plant material

DNA material of one hundred and three accessions (Table 1) from Kardolus (1998), representing 47 tuber-bearing wild and cultivated *Solanum* species plus two outgroup species, was used for NBS profiling. Origin of the material and number of genotypes per accession are given in Table 1. To facilitate comparison with the previous results from Kardolus (1998), we used the series and species abbreviations according to Hawkes (1990), although we are aware of the synonymy published since then.

### NBS profiling procedure

NBS profiling was performed essentially as described by van der Linden et al. (2004) with two minor modifications. Firstly, 200 instead of 400 ng of DNA was digested with a restriction enzyme. Secondly, in the study of van der Linden et al. (2004), the restriction and adaptor ligation reactions were done separately, while in our study, the two were combined into one single reaction of 60 µl, consisting of 200 ng of DNA, 30 pmol of adaptor, 60 mM ATP, 10 U enzyme, 5 U T4 ligase and restriction and ligation buffer (10 mM Tris.HAc pH7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA). Afterwards, the restriction/ligation product was two times diluted with MilliQ water and used as template for PCR reactions.

**Table 1** Accessions used for phylogeny reconstruction

| Code[a] | Series | Species | Origin | Source [b] | Chr no | # GT [c] |
|---|---|---|---|---|---|---|
| ach1 | TUB | *S. achacachense* Cárdenas | Bolivia | B29617 | 24 | 10 |
| acl7 | ACA | *S. acaule* Bitter ssp. *acaule* | Bolivia | B28026 | 48 | 10 |
| acl10 | ACA | *S. acaule* ssp. *acaule* | Bolivia | B27206 | 48 | 10 |
| acl12 | ACA | *S. acaule* ssp. *acaule* | Bolivia | B27361 | 48 | 10 |
| acl13 | ACA | *S. acaule* ssp. *acaule* | Argentina | B16835 | 48 | 10 |
| acl18 | ACA | *S. acaule* ssp. *acaule* | Argentina | B17111 | 48 | 10 |
| acl26 | ACA | *S. acaule* ssp. *acaule* | Argentina | B47627 | 48 | 10 |
| acl27 | ACA | *S. acaule ssp. acaule* | Argentina | B17181 | 48 | 10 |
| adg1 | TUB | *S. tuberosum* L. ssp. *andigena* Hawkes | - | B7462 | 48 | 10 |
| adg2 | TUB | *S. tuberosum* ssp. *andigena* | - | B24677 | 48 | 9 |
| aem1 | ACA | *S. acaule* ssp. *aemulans* Hawkes et Hjert. | Argentina | B17129 | 48 | 10 |
| aem5 | ACA | *S. acaule* ssp. *aemulans* | Argentina | PI320280 | 48 | 10 |
| ajh1 | TUB | *S. ajanhuiri* Juz. et Bukasov | - | CIP702677 | 24 | 10 |
| alb2 | ACA | *S. albicans* Ochoa | Peru | CIP761438 | 72 | 1 |
| alb3 | ACA | *S. albicans* | Peru | PI365376 | 72 | 10 |
| alb5 | ACA | *S. albicans* | Peru | PI498194 | 72 | 10 |
| bcp1 | DEM | *S. brachycarpum* Corr. | Mexico | B8100 | 72 | 10 |
| ber1 | TUB | *S. berthaultii* Hawkes | Bolivia | B28009 | 24 | 9 |
| ber2 | TUB | *S. berthaultii* | Bolivia | B24578 | 24 | 10 |
| ber3 | TUB | *S. berthaultii* | Bolivia | B10063 | 24 | 10 |
| blb | BUL | *S. bulbocastanum* Dunal | Mexico | B8009 | 24 | 3 |
| blv3 | MEG | *S. boliviense* Dunal | Bolivia | B27342 | 24 | 10 |
| brc1 | TUB | *S. brevicaule* Bitter | Bolivia | B18291 | 24 | 10 |
| brc2 | TUB | *S. brevicaule* | Bolivia | B28023 | 24 | 9 |
| brd2 | ETU | *S. brevidens* Phil. | Argentina | B17441 | 24 | 10 |
| bst1 | PIN | *S. brachistotrichium* (Bitt.) Rydb. | Mexico | B7986 | 24 | 10 |
| bst2 | PIN | *S. brachistotrichium* | Mexico | B7987 | 24 | 10 |
| buk1 | TUB | *S. bukasovii* Juz. | Peru | B15424 | 24 | 10 |
| buk2 | TUB | *S. bukasovii* | Peru | B18294 | 24 | 10 |
| can1 | TUB | *S. canasense* Hawkes | Peru | B8105 | 24 | 10 |
| can2 | TUB | *S. canasense* | Peru | B7162 | 24 | 8 |
| can3 | TUB | *S. canasense* | Peru | B8012 | 24 | 9 |
| cha1 | TUB | *S. chaucha* Juz. et Bukasov | - | CIP 700145 | 36 | 1 |
| cha4 | TUB | *S. chaucha* | - | CIP701568 | 36 | 1 |
| chc1 | YNG | *S. chacoense* Biter ssp. *chacoense* | Argentina | B17034 | 24 | 10 |
| chc2 | YNG | *S. chacoense* ssp. *chacoense* | Argentina | B17018 | 24 | 7 |
| cop1 | TUB | *S. coelestipetalum* Vargas | Peru | B7942 | 24 | 7 |
| cop3 | TUB | *S. coelestipetalum* | Peru | B7994 | 24 | 10 |
| crc | CIR | *S. circaeifolium* Bitter ssp. *circaeifolium* | Bolivia | B27058 | 24 | 10 |
| dms1 | DEM | *S. demissum* Lindl. | Mexico | B10030 | 72 | 10 |
| dms2 | DEM | *S. demissum* | Mexico | B10022 | 72 | 10 |
| dms3 | DEM | *S. demissum* | Mexico | B9990 | 72 | 10 |
| etb1 | ETU | *S. etuberosum* Lindl. | Chili | B28476 | 24 | 9 |
| etb2 | ETU | *S. etuberosum* | Chili | B8082 | 24 | 10 |
| fen | LON | *S. fendleri* A. Gray ssp. *fendleri* | Mexico | B7230 | 48 | 9 |
| gnd | TUB | *S. gandarillasii* Cárdenas | Bolivia | B7174 | 24 | 9 |

| Code[a] | Series | Species | Origin | Source [b] | Chr no | # GT [c] |
|---|---|---|---|---|---|---|
| grl2 | TUB | *S. gourlayi* Hawkes ssp. *gourlayi* | Argentina | B17338 | 24 | 9 |
| grl4 | TUB | *S. gourlayi* ssp. *gourlayi* | Argentina | B16837 | 48 | 10 |
| hje | LON | *S. hjertingii* Hawkes | Mexico | B8088 | 48 | 9 |
| ifd1 | CUN | *S. infundibuliforme* Phil. | Argentina | B17212 | 24 | 4 |
| juz3 | TUB | *S. juzepczukii* Bukasov | - | CIP701895 | 36 | 1 |
| ktz1 | TUB | *S. kurtzianum* Bitter et Wittm. | Argentina | B17585 | 24 | 10 |
| ktz2 | TUB | *S. kurtzianum* | Argentina | B16861 | 24 | 10 |
| ktz3 | TUB | *S. kurtzianum* | Argentina | B17580 | 24 | 10 |
| les | POL | *S. lesteri* Hawkes et Hjert. | Mexico | B55219 | 24 | 10 |
| lgl | LIG | *S. lignicaule* Vargas | Peru | B8106 | 24 | 12 |
| lph1 | TUB | *S. leptophyes* Bitter | Argentina | B7184 | 24 | 8 |
| lph2 | TUB | *S. leptophyes* | Bolivia | B27176 | 24 | 10 |
| lph3 | TUB | *S. leptophyes* | Bolivia | B27211 | 24 | 10 |
| mcd1 | TUB | *S. microdontum* Bitter | Bolivia | B31189 | 24 | 10 |
| mcd2 | TUB | *S. microdontum* | Argentina | B24649 | 24 | 10 |
| mcq1 | TUB | *S. mochiquense* Ochoa | Peru | B32672 | 24 | 8 |
| mcq2 | TUB | *S. mochiquense* | Peru | B8142 | 24 | 10 |
| mga3 | MEG | *S. megistacrolobum* Bitter ssp. *megistacrolobum* | Argentina | B17642 | 24 | 10 |
| mgl | MGL | *S. maglia* Schlechtd. | Chili | B23571 | 24 | 10 |
| mlt1 | TUB | *S. multidissectum* Hawkes | Peru | B8145 | 24 | 10 |
| opl1 | TUB | *S. oplocense* Hawkes | Argentina | B16868 | 72 | 10 |
| opl2 | TUB | *S. oplocense* | Argentina | B24650 | 72 | 10 |
| opl3 | TUB | *S. oplocense* | Argentina | B16879 | 72 | 10 |
| oxc | CON | *S. oxycarpum* Schiede | Mexico | B53011 | 48 | 10 |
| pcs1 | PIU | *S. paucissectum* Ochoa | Peru | B8162 | 24 | 6 |
| pcs2 | PIU | *S. paucissectum* | Peru | B55216 | 24 | 10 |
| phu1 | TUB | *S. phureja* Juz. et Bukasov | - | B15482 | 24 | 10 |
| phu2 | TUB | *S. phureja* | - | B50199 | 24 | 10 |
| pne1 | ACA | *S. acaule* ssp. *punae* Hawkes et Hjert. | Peru | PI365312 | 48 | 10 |
| pne2 | ACA | *S. acaule* ssp. *punae* | Peru | B7958 | 48 | 10 |
| pne4 | ACA | *S. acaule* ssp. *punae* | Peru | PI473442 | 48 | 10 |
| pnt1 | PIN | *S. pinnatisectum* Dunal | Mexico | B8168 | 24 | 10 |
| qum | CIR | *S. circaeifolium* ssp. *quimense* Hawkes et Hjert. | Bolivia | B27034 | 24 | 10 |
| rap1 | MEG | *S. raphanifolium* Cárdenas et Hawkes | Peru | B15445 | 24 | 10 |
| rap3 | MEG | *S. raphanifolium* | Peru | B7207 | 24 | 10 |
| sct1 | MEG | *S. sanctae-rosae* Hawkes | Argentina | B15454 | 24 | 10 |
| sct2 | MEG | *S. sanctae-rosae* | Argentina | B17568 | 24 | 7 |
| sct3 | MEG | *S. sanctae-rosae* | Argentina | B17051 | 24 | 10 |
| spg2 | TUB | *S. spegazzinii* Bitter | Argentina | B24694 | 24 | 8 |
| spg3 | TUB | *S. spegazzinii* | Argentina | B16905 | 24 | 10 |
| spl1 | TUB | *S. sparsipilum* (Bitt.) Juz. et Bukasov | Bolivia | B8209 | 24 | 10 |
| spl2 | TUB | *S. sparsipilum* | Bolivia | B8150 | 24 | 10 |
| spl3 | TUB | *S. sparsipilum* | Bolivia | B15455 | 24 | 10 |
| stn1 | TUB | *S. stenotomum* Juz. et Bukasov ssp. *stenotomum* | Bolivia | B27165 | 24 | 10 |
| stn2 | TUB | *S. stenotomum* ssp. *goniocalyx* Hawkes | Peru | B7478 | 24 | 10 |
| sto | LON | *S. stoloniferum* Schlechtd. et Bche. | Mexico | B7229 | 48 | 10 |
| tar1 | YNG | *S. tarijense* Hawkes | Argentina | B17423 | 24 | 10 |
| tar2 | YNG | *S. tarijense* | Argentina | B8229 | 24 | 6 |

| Code[a] | Series | Species | Origin | Source [b] | Chr no | # GT [c] |
|---|---|---|---|---|---|---|
| tbr1 | TUB | *S. tuberosum* L. ssp. *tuberosum* | - | 'Certa' x 'Gloria' | 48 | 1 |
| ver1 | TUB | *S. verrucosum* Schlechtd. | Mexico | B8255 | 24 | 10 |
| ver2 | TUB | *S. verrucosum* | Mexico | B8246 | 24 | 7 |
| ver3 | TUB | *S. verrucosum* | Mexico | B8254 | 24 | 4 |
| vid1 | TUB | *S. gourlayi* ssp. *vidaurrei* Hawkes et Hjert. | Argentina | B16831 | 24 | 10 |
| vid2 | TUB | *S. gourlayi* ssp. *vidaurrei* | Argentina | B18528 | 24 | 9 |
| vrn1 | TUB | *S. vernei* Bitter et Wittm. | Argentina | B15451 | 24 | 8 |
| vrn2 | TUB | *S. vernei* ssp. *ballsii* Hawkes et Hjert. | Argentina | B17536 | 24 | 10 |
| vrn3 | TUB | *S. vernei* ssp. *vernei* Bitter et Wittm. | Argentina | B17542 | 24 | 10 |

[a] Series and taxon code abbreviations according to Hawkes (1990)

[b] Accessions with a prefix of B were obtained from the Braunschweig Genetic Resources Collection; Accessions with prefix CIP were obtained from the International Potato Centre, Peru; Accessions with prefix PI showed the Plant Introduction Number after Bamberg et al. (1996)

[c] Number of genotypes used to represent the accession in the DNA sample

The 25 µl PCR reaction mix consisted of 5 µl template DNA, 20 pmol of both adaptor primer and NBS primer, 200 µM dNTPs, 0.4 U HotStarTaq (Qiagen, Germany), and 2.5 µl HotStarTaq PCR buffer. The PCR program consisted of 30 cycles of 30 s at 95°C, 1 min 40 s at 55–60°C annealing, and 2 min at 72°C. Annealing temperature was 55°C for NBS5 and NBS9 primers and 60°C for NBS2 and NBS3 primers. After this, a second PCR reaction was performed using 5 µl of the 10 times diluted first PCR product as template, and the same NBS-specific primer but now $^{33}$P radioactively labeled, and cycling conditions similar to the first PCR. Labeled products were separated on 6% polyacrylamide gels. X-ray films were exposed to the gels to visualize individual fragments. The presence or absence of polymorphic fragments was scored on the autoradiograms and transferred into a 1 (present) and 0 (absent) binary matrix for all accessions.

In total, four primers (NBS2, NBS3, NBS5 and NBS 9) and three enzymes (*MseI*, *RsaI* and *AluI*) were used (Table 2). NBS2, NBS5 and adaptor sequences were the same as described by van der Linden et al. (2004). NBS3 and NBS9 primer sequences were as follows: NBS3 5'- GTWGTYTTICCYRAICCIGGCATICC-3' and NBS9 5'-TGTGGAGGRTTACCTCTAGC-3'. Positions of the NBS primers in the NBS domain are shown in Fig.1.

**Table 2** Number of polymorphic NBS markers for different primer/enzyme combinations. Bottom row shows RGA content for the four primers.

| Enzyme/Primer | NBS5 | NBS9 | NBS2 | NBS3 |
|---|---|---|---|---|
| MseI | 32 | 36 | 66 | 37 |
| Rsa | 31 | 23 | 29 | ND[a] |
| AluI | 37 | 40 | ND | 31 |
| RGA content (%) | 94 | 57 | 63 | 61 |

[a] ND: no data

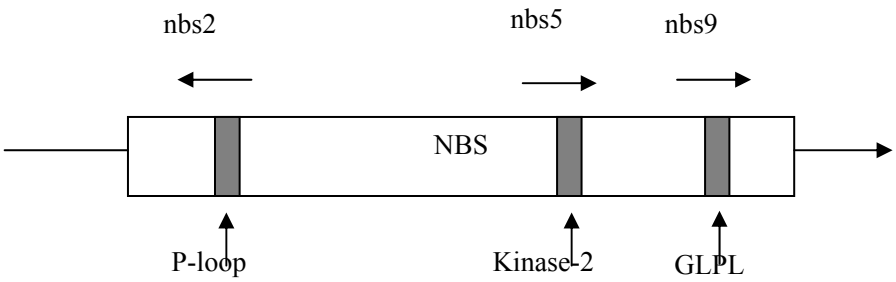


**Fig. 1** Schematic representation of the nucleotide-binding site (NBS) of disease resistance genes and positions of primers used in this study. P-loop, kinase-2 and GLPL refer to highly conserved motifs in the NBS. LRR refers to the Leucine Rich Repeat. Primer positions are indicated by horizontal arrows.

**Annotation of NBS profiling sequences**

To determine the RGA content of NBS profiling markers, 384 bands were excised from the gel, re-amplified with PCR conditions identical to the first PCR of the NBS profiling protocol and purified with Qiaquick PCR purification spin columns (Qiagen). Fragments were directly sequenced using the adaptor primer as a sequencing primer with the BigDye Terminator kit and an ABI 3700 automated sequencer from Applied Biosystems (USA). Sequences of poor quality were excluded from further analysis, and the remaining sequences were compared to archived sequences in the NCBI nucleotide databases (Jan 11, 2006) using BLASTN and BLASTX (Altschul et al. 1997). Sequences that were significantly similar to known R genes and RGAs (either E value lower than 1e-05 for BLASTN, or E value lower than 1e-03 for BLASTX) were regarded as RGAs.

### NBS profiling and AFLP data analysis

AFLP data from three primer combinations were available from Kardolus (1998). Two AFLP datasets E32M49, E35M48 and one NBS profiling dataset were used in this study. Neighbor joining (NJ) and parsimony analyses were performed with PAUP* version 4.0 b10 (Swofford 2001). For the parsimony analysis, we used a two-step search strategy. (1) The first heuristic search was conducted with 10,000 random additions, holding 10 trees, and saving five trees per search replicate. (2) Resulting trees from the first search were used as starting trees to swap to completion with MULTREES and TBR. A jackknife analysis (10,000 replicates) was performed with the same settings as the heuristic search. In the parsimony analyses, three accessions from the series *Etuberosa* Juz. were used as outgroups.

Congruence between AFLP and NBS profiling was assessed in three ways: (1) visual qualitative comparisons of the trees obtained, (2) distance matrix-based comparisons, and (3) character-based comparisons.

For the distance matrix-based comparisons, the program SIMQUAL was used to compute similarity matrices using the DICE option, which ignores shared absent bands, and is an appropriate algorithm for dominant markers like AFLP or NBS profiling markers. A pair wise comparison for these matrices with the Mantel test in NTSYS-pc version 2.10j (Rohlf 1992) was made. For the character-based comparisons, the partition homogeneity test (Farris et al. 1995) was performed in PAUP* version 4.0 b10 (Swofford 2001). This test is also called the incongruence length difference (ILD) test. ILD tests were performed with 100 replicates, the heuristic search option, and TBR and MULPARS in effect.

## Results

### NBS profiling on *Solanum* species

NBS profiling produced well scorable banding patterns on a gel; part of such a gel is shown in Fig. 2. For the whole set of 103 accessions, ten primer/enzyme combinations were tested. A dataset of 362 characters was produced. Each combination produced 23-66 scorable polymorphic bands (Table 2).

To obtain information on the RGA content of the bands that make up the NBS profiles,

384 bands were sequenced, of which 232 produced readable sequences. Table 2 shows the percentage of RGA-derived bands for each primer. The RGA content of the bands obtained with the NBS5 primer was 94%. For NBS9, NBS2 and NBS3, these figures were 57%, 63% and 61%, respectively. For all primer combinations together, the average RGA content was 67%, indicating that NBS profiles indeed largely consisted of markers derived from R-genes and their analogs. Some examples of bands that could be annotated are listed in Table 3. Most of the remaining fragments (33%) could not be annotated. Only in a few cases significant homologies to other known genes, like retrotransposons, were found.

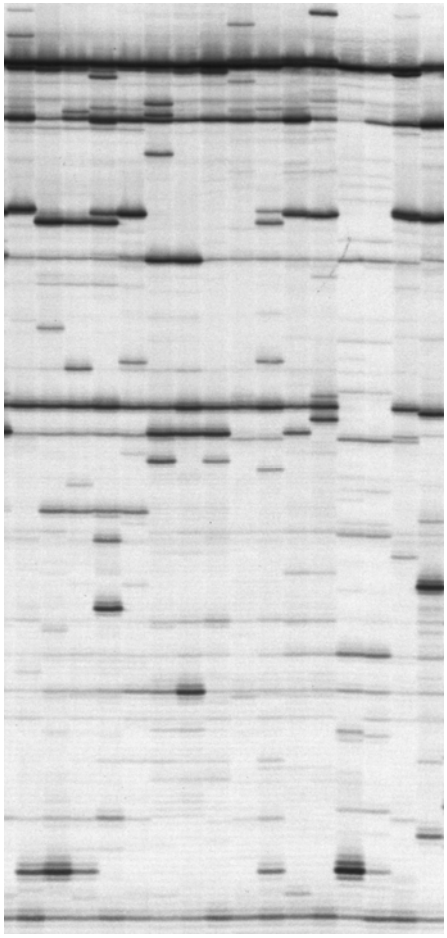

**Fig. 2** Part of an NBS profiling gel obtained with primer NBS9 and restriction enzyme *MseI*.

**Cladistic results**

The NBS profiling dataset was composed of 362 characters, 351 of which were parsimony informative. Sixteen most parsimonious 3,553-step trees were produced, with a consistency index of 0.102 and retention index of 0.464. The strict consensus tree is shown in Fig. 3. Numbers above the branches indicated jackknife values >50%.

The three non-tuber-bearing *Solanum* species (outgroup species: brd2, etb1 and etb2) were basal to the tree. Next came a polytomy consisting of (1) the diploid Mexican species (bst1, bst2, blb, les, pnt1), (2) diploid South American species (mcq1, mcq2, pcs1, pcs2), (3) the representatives of series *Circaeifolia* (crc and qum), (4) the species *S. lignicaule*, and (5) all other species. Within the latter group four clades could be distinguished but none of

them had statistical support: a group of Mexican polyploids including the diploid *S. verrucosum*, a group consisting of taxa from series *Acaulia*, including *S. demissum* from series *Demissa*, with a number of species of series *Megistacroloba* as a sister clade, and two groups with mainly species from series *Tuberosa*, divided in a Bolivian and Peruvian group, the latter also containing a number of cultivated species. Only the *Acaulia* group had a high jackknife support of 99%.

The AFLP datasets resulting from the two chosen primer combinations were found to be congruent with each other both for the Mantel test and ILD test (data not shown). For this reason we combined the two AFLP datasets for the comparison with NBS profiling.

The AFLP dataset consisted of 591 characters, among which 539 were informative. Parsimony analysis of AFLP data yielded eight equally parsimonious 1,548-step trees with a consistency index of 0.169 and retention index of 0.542. The strict consensus tree is shown in Fig. 4.

The outgroup species are again at the base of the tree, followed by the Mexican diploids, the taxa of series *Circaeifolia*, and relatively primitive South American species.

The remainder of the tree was subdivided into three clades: a group of Peruvian species of series *Tuberosa* with the accessions of the cultivated species, a group of taxa from the series *Acaulia*, including *S. demissum*, forming a polytomy with a number of species from the series *Megistacroloba*, and a large polytomy including mainly the Bolivian representatives of series *Tuberosa*, with the Mexican polyploids nested within.

**Table 3** NBS profiling bands with high similarity to known resistant (R) genes and R gene cluster members

| Band Code | Primer | Homologue | Identity (no.of nucleotides) | E value for BLASTN |
|---|---|---|---|---|
| 65 | nbs2 | *Solanum tuberosum* potato resistance-like protein I2GA-SH23-3 | 90% (324) | 5.00E-82 |
| 1 | nbs2 | *Lycopersicon esculentum* BAC clone Clemson_Id 127E11 | 92% (455) | 2.00E-162 |
| g0739 mq21 | nbs5 | *Solanum bulbocastum* Rpi-blb2 gene | 97% (281) | 8.00E-137 |
| g0732 mq14 | nbs5 | *Solanum demissum* chromosome 11 clone PGEC591C22 map MAP_LOC | 96% (457) | 0 |
| G14 | nbs9 | *Lycopersicon esculentum* Tm-2 ToMV resistant protein (Tm-2nv) gene | 92% (276) | 7.00E-97 |
| 18c-7 | nbs9 | *Solanum acaule* Rx2.ac15 gene | 97% (223) | 1.00E-93 |

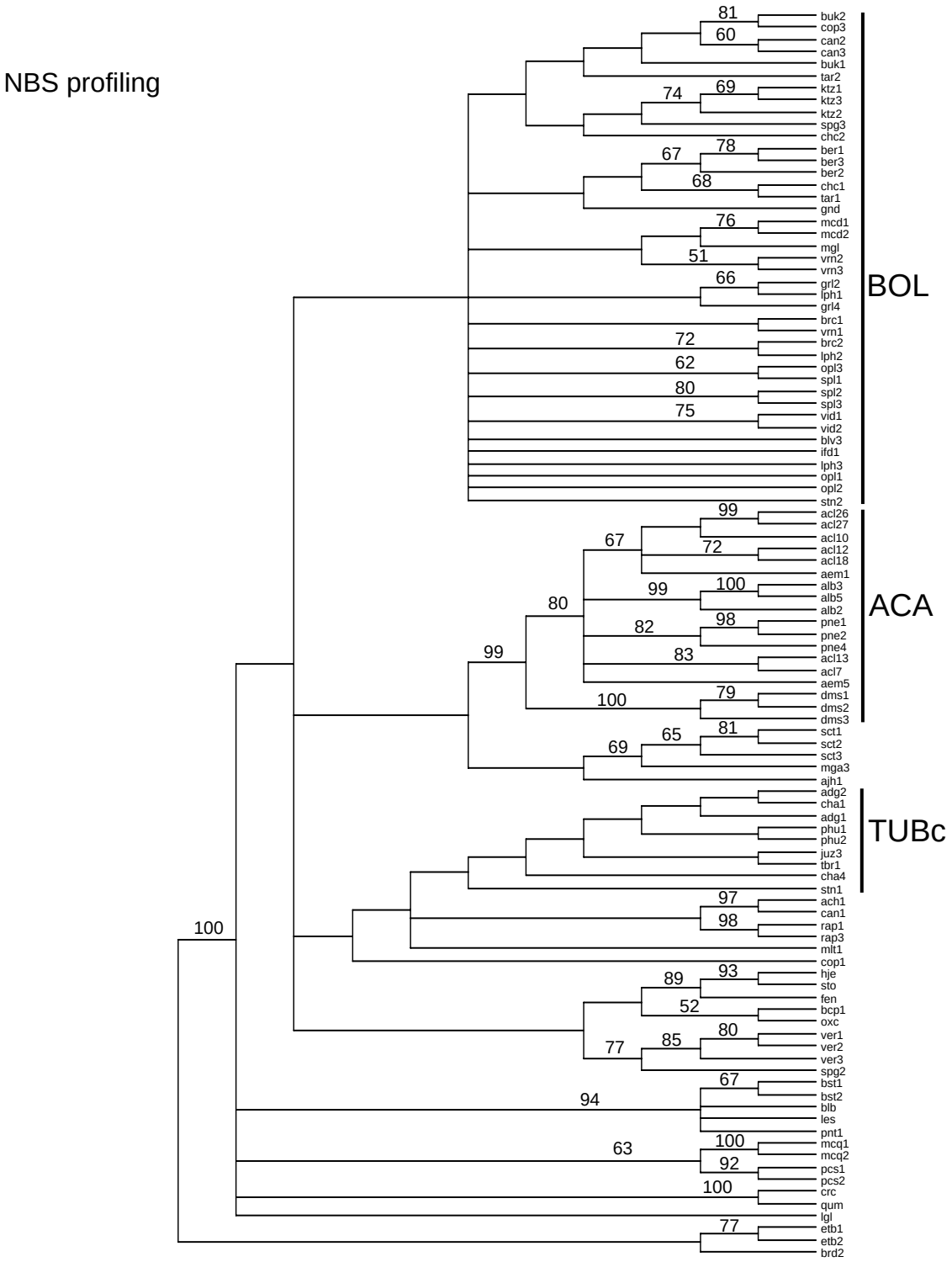


**Fig. 3** NBS profiling strict consensus tree. Numbers above the branches are jackknife supports based on 10,000 replicates (%). Jackknife support values lower than 50% are not shown. ACA and TUB are abbreviations for series *Acaulia* and *Tuberosa* outlined by Hawkes (1990). Clade ACA includes a group of taxa from series *Acaulia* together with *S. demissum* from series *Demissa*. TUBc indicates the cultivated species from series *Tuberosa*. BOL includes accessions from series *Tuberosa* from Bolivia, Argentina and Chile.

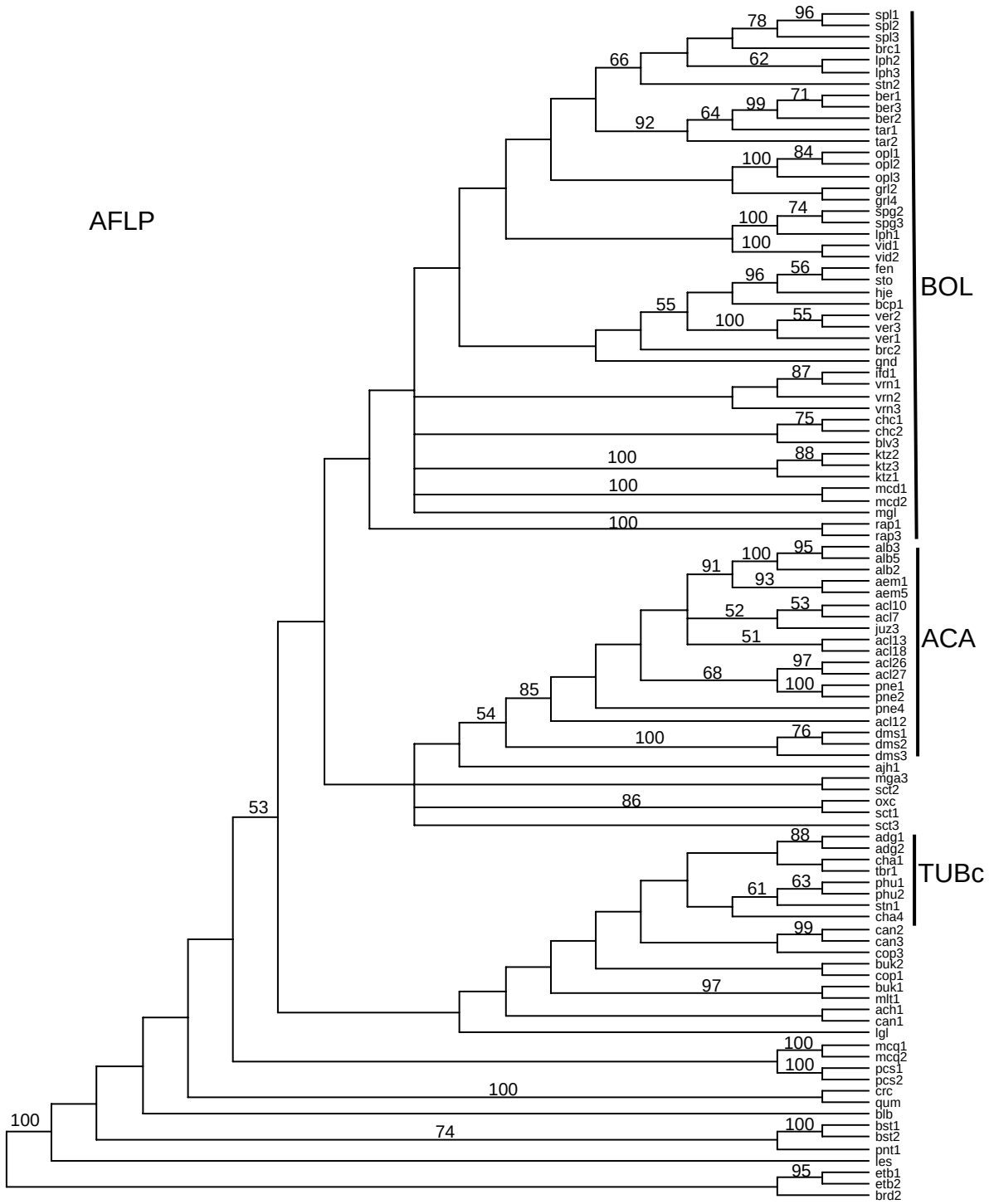


**Fig. 4** AFLP strict consensus tree. For details see figure 3.

In both the NBS profiling and AFLP based strict consensus trees, the more basal branches either had poor jackknife support (lower than 50%) or form a polytomy. To investigate the influence of polyploid taxa on the poor resolution of the trees, we excluded all polyploids from the analysis. Two new datasets, containing the marker data of 72 accessions from the diploid species were produced. Parsimony and jackknife analysis were performed in the same way as with the complete dataset. Excluding the polyploid species did not improve the trees, the polytomies and basal nodes with jackknife support lower than 50% were still present.

At the lower (accession) level, common well supported subclades can be found in both the NBS profiling and AFLP strict consensus trees. In total, seventeen subclades (containing two or three accessions) with a jackknife support higher than 70% were observed (Table 4).

**Table 4** Common groups recognized both from AFLP and NBS profiling tree

| Group | Common Groups | NBS Profiling Jackknife(%) | AFLP Jackknife(%) |
|---|---|---|---|
| 1 | acl 26 acl27 | 99 | 97 |
| 2 | alb2 3 5 | 99 | 100 |
| 3 | alb3 alb5 | 100 | 95 |
| 4 | ber1 ber3 | 78 | 71 |
| 5 | crc qum | 100 | 100 |
| 6 | dms1 dms2 | 79 | 76 |
| 7 | dms1 2 3 | 100 | 100 |
| 8 | etb1 etb2 | 77 | 95 |
| 9 | hje sto fen | 89 | 96 |
| 10 | ktz1 2 3 | 74 | 100 |
| 11 | mcd1 mcd2 | 76 | 100 |
| 12 | mcq1 mcq2 | 100 | 100 |
| 13 | pcs1 pcs2 | 92 | 100 |
| 14 | pne1 pne2 | 98 | 100 |
| 15 | rap1 rap3 | 98 | 100 |
| 16 | ver1 2 3 | 85 | 100 |
| 17 | vid1 vid2 | 75 | 100 |

**Phenetic results**

NBS profiling and AFLP phenograms had a very high cophenetic correlation coefficient of 0.96 and 0.93, indicating excellent fits of the similarity matrices to the resulting

phenograms. The NJ trees of both NBS profiling and AFLP data basically showed the same groups as the cladograms. Except for the branch leading to the outgroups many of the interconnecting branches were very short and jackknife support was low. Average similarities for AFLP and NBS-profiling were 0.29 and 0.52, respectively.

### Congruence between AFLP and NBS profiling derived data

As described above, a visual inspection of the trees produced from NBS profiling and AFLP data showed that they were similar in many aspects, although many details were different. The correlation coefficient between NBS profiling and AFLP similarity matrices was 0.737, and between the cophenetic value matrices derived from the NBS profiling and AFLP trees 0.762. Mantel test showed that both were significantly correlated at $\alpha$=0.05 (P=0.001), indicating both NBS profiling and AFLP derived similarity matrices and tree topologies are congruent.

In contrast to the Mantel test, the ILD test produced different results. P value of 0.01 was observed, showing that NBS profiling and AFLP were incongruent with each other ($\alpha$=0.05).

## Discussion

### NBS profiling in *Solanum*

When introducing a new tool for systematic analysis one first needs to compare the results from the new tool with results from established techniques, like AFLP (Vos et al. 1995; Kardolus et al. 1995). Our study aimed at such comparison. We first investigated whether NBS profiling produces markers derived from resistance genes and their analogs with a high frequency. Table 2 clearly shows that the majority of the bands were derived from resistance genes and their analogs. Most likely, the observed RGA content is even an underestimation as bands can only be identified when sufficiently homologous sequences are present in the nucleotide databases. This was supported by the observation that some bands could not be annotated during our first analysis in August 2004, while the second analysis, with an updated version of the database (in January 2006), yielded a positive identification for several previously unidentifiable bands. Also, the annotation became more accurate. An example was the band g0739 mq21 obtained with the primer NBS5 in

the *S. bulbocastanum* BGRC 8009 (Table 3). The first analysis showed that this band had the highest similarity with the *Mi*-gene of tomato. During the second annotation round, this fragment had the highest similarity (97%) with the late blight resistance gene rpi-blb2 (van der Vossen et al. 2005).

**Systematic relationships inferred from NBS profiling and AFLP**

The topology of the cladogram from our AFLP analysis is comparable to Kardolus (1998), which is not surprising since we studied a subset of his material (because not all of the DNA was still available). Kardolus (1998) distinguished more groups in his trees but also in his results the Mexican diploid species and primitive South American species like *S. circaeifolium* and *S. mochiqense* are placed in a basal group. Furthermore, a distinct group of representatives of series *Acaulia* can be recognized, closely related to the Mexican hexaploid species *S. demissum*, and the species belonging to the series *Tuberosa* are separated in a Peruvian (including the cultivated material) and a Bolivian group. He only showed bootstrap support values for the groups in his NJ tree, and generally the values for deeper branches are lower than 50%. The pattern from our analyses of the NBS profiling dataset is similar to that of Kardolus (1998).

This general pattern in our AFLP and NBS profiling results supplements the results of the most extensive cpDNA RFLP study of section *Petota* (Spooner et al. 1997). They recovered four clades, with representatives of the Mexican diploids in clade 1 and 2, species like *S. mochicense* and *S. paucissectum* in clade 3, and a large polytomy of the other investigated species in clade 4. Spooner et al. (2005a) performed phylogenetic analyses of AFLP data of 362 individual wild (261) and landrace (98) members of section *Petota*. The strict consensus tree also has bootstrap values lower than 50% for the deeper branches (except the designated outgroup). In rDNA ETS results (Volkov et al. 2003) only three structural variants are found, with variant A present in the non-tuber-bearing species of series *Etuberosa* and in the representatives of the Mexican diploid series, variant B in series *Circaeifolia*, and variant C in all other investigated species. The dendrograms presented show many polytomies, indicating that resolution within the groups is mostly lacking.

When comparing the cladograms from AFLP and NBS profiling data (Figure 3 and 4) visually, there were congruencies at the lower level with accessions grouping together in the same way in both trees. These groups were supported with jackknife values >70% (Table 4). When looking at the more basal nodes, we found only few groups (ACA) which were well supported by relatively high jackknife values. A number of other groups were present, but they lacked statistical support. Again this was similar for both trees. Two different statistical tests were used to evaluate the congruency between the AFLP and NBS profiling based trees. The outcome of the tests was different. Whereas congruency was indicated by the Mantel test, the ILD test indicated incongruency. Similar observations were reported by Spooner et al. (2005b), who compared AFLPs with other markers for phylogenetic inference in wild tomatoes. In their Mantel test, the comparison of cpDNA/GBSSI (granule-bound starch synthase gene) gives a high matrix correlation coefficient (0.831), but fails to pass the ILD test. The suitability of the ILD test is also questioned by other researchers (Graham et al. 1998; Yoder et al. 2001; Barker et al. 2002; Darlu et al. 2002).

The interesting observation that the overall pairwise similarity based on NBS profiling markers was 25 % higher than the overall similarity based on AFLP markers, suggests that NBS profiling markers are more conserved than AFLP markers. Targeting more conserved regions renders the marker system more appropriate than AFLP when materials are more diverse, as it will reduce the chance for homoplasy.

The fact that the basal branches either had poor jackknife support (lower than 50%) or formed a polytomy in both the AFLP and NBS profiling tree, probably results from the nature of the studied material. Apparently, *Solanum* species are more similar to each other than expected on the basis of morphological characters, even when the polyploid species were excluded from the analysis. Extensive hybridization and introgression among species might be the reason for the poor resolution at the more basal nodes. An alternative explanation could be that many *Solanum* species have evolved in a relatively short period of time after rapid radiation over South America, which would result in species with distinct characters but with no clear sequential branching order (apart from originating from the common ancestor).

## The role of disease resistance in speciation

Many NBS profiling markers were shown to be RGA-related (Table 2). NBS profiling preferentially generates markers in resistance genes that are likely to be under selection, which might influence the outcome of the phylogenetic analysis. Disease resistance might play a role in the speciation process and it may thus be challenged whether NBS profiling can be used for phylogeny reconstruction. Our results demonstrated that systematic relationships from NBS profiling data do not essentially differ from that from AFLP data. This congruence between AFLP and NBS profiling may not be as unexpected as it appears to be. Plants have to deal with many different pathogens during their lifetime and are thus exposed to selective pressures in different directions. This is also evidenced by the large number of NBS-LRR resistance genes present in plants. However, it is possible that a single resistance gene was essential for species survival, or a speciation event. The R-gene(s) that may have been under selection after a period of disease pressure by a pathogen may have spread relatively fast in neighboring species through hybridization. The specific effect of selective pressure on R-genes will therefore be only detectable on a very short evolutionary time scale, and is diluted when many markers are analyzed phylogenetically. In addition, the selective advantage of retaining a specific R-gene will most likely be reflected by the absence or presence of one or a few markers in NBS profiling. The outcome of the phylogenetic analysis is based on all NBS profiling markers, the majority of which was not affected by selective pressure. A single or a few markers very likely will not influence the outcome.

In conclusion, NBS profiling is at least as good as AFLP for phylogeny reconstruction and might even be superior when more diverse material is used, as it will reduce the chance for homoplasy. The observation that higher level groups were poorly supported might be due to the nature of the material and the way the species evolved.

## Acknowledgements

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# Chapter 3
# Genetic diversity in a large set of European potato varieties and identification of resistance gene markers in varieties and their relatives using NBS profiling

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**Abstract**

In this study we assessed the changes in genetic diversity during the last 70 - 80 years in a set of 456 European potato varieties using NBS profiling. Analysis showed that during that period diversity in markers linked to resistance had increased. Only one marker was lost, whereas several new markers were introduced into the potato gene pool. This most likely reflects the efforts of breeding companies and research institutes to introgress resistances from wild species into cultivated potato. NBS profiling was also used to identify candidate resistance gene markers. Several markers, potentially linked to introgressed R-genes, are identified by linking NBS profiling bands with pedigree data and phenotypic data of the varieties. It is also possible to link these markers to wild tuber-bearing *Solanum* species as homoplasy in NBS profiling markers turned out to be low. Co-migrating NBS profile marker bands were sequenced to check the identity of the bands. Within the tuber-bearing *Solanum* species, 91% of the sequences of co-migrating bands were more than 95% homologues to each other, indicating low levels of homoplasy. One of the resistance markers identified was most likely introgressed from *Solanum vernei*. This is indicated by the correlation between the presence of this marker and the resistance data for the varieties involved, as well as the presence of the same marker in both *S. vernei* accessions examined.

## Introduction

The cultivated potato, *Solanum tuberosum* subsp. *tuberosum* (2n = 4x = 48), is one of the most important food crops in the world. To ensure sustainable potato production in a changing environment, continuous genetic improvement of the cultivated potato is essential.

Varieties used in North America and Europe are derived from a small number of founding varieties. A study on North American varieties showed that, based on the 1996 certified seed acreage, 46 varieties could be identified as founding clones and major contributing ancestors (Love 1999). Similarly, the majority of the European cultivated potato varieties originate from around 30 varieties that were introduced in the nineteenth century, as indicated by the pedigree database (Hutten and van Berloo 2001). It is interesting to see whether the genetic diversity of European potato varieties has been affected by breeding during the last century. This question is particularly interesting with respect to disease resistance. Breeding for resistance towards several diseases has been a focus in potato and has resulted in the introduction of new genetic variation from wild relatives. For instance, late blight resistance was introduced in cultivated potato around 1950, with *S. demissum* as a major donor species. Potato Cyst Nematode (PCN) resistance was introduced into potato varieties in the late 1960s, for example through the *S. tuberosum* subsp. *andigena* accession CPC 1673, which confers resistance not only to the cyst nematode *Globodera pallida* (Rouppe van der voort et al. 1997) but also to potato virus X (Bendahmane et al. 1997). This accession has been ancestor to many potato varieties, for example, ‘Sante’.

On the other hand, the selection for a limited set of traits may have resulted in the loss of important alleles, which increase vulnerability of the cultivated potato for new/other diseases. Genetic diversity at resistance loci can be assessed using NBS profiling (van der Linden et al. 2004). NBS profiling is an efficient and reproducible molecular marker system that specifically targets resistance genes (R genes) and their analogs (RGAs) (van der Linden et al. 2004; Calenge et al. 2005).

For many European potato varieties, pedigree records for the past 30 years are available, which makes it possible to trace the sources of introgression. By linking phenotypic, pedigree and marker data, it may be possible to identify candidate R-gene

markers. Several approaches can be used to validate these candidate resistance gene markers: through segregating populations (Michelmore et al. 1991) by association mapping (Malosetti et al.2007; Gebhardt et al. 2004), or by confirming the presence of the marker in the predicted wild species.

This study examines the genetic diversity changes over the last 70-80 years in resistance related markers using a large set of potato varieties. In addition, it explores the possibilities to link molecular markers to specific resistance traits by combining NBS profiling data with the pedigree and phenotypic data. Using this approach, several markers are identified that are potentially linked to introgressed R-genes. Finally, we extended our study (chapter 2) to examine the utility of NBS profiling for systematics by addressing the sequence homology of co-migrating NBS profile bands.

## Materials and Methods

### Plant material, phenotypic and pedigree data

Four hundred and fifty six potato varieties were collected from various sources. Those originating form the United Kingdom were obtained from morphologically verified tubers from the NIAB potato collection, or from leaves of potato plants in the SASA potato collection. Dutch and German varieties were obtained from four sources: (1) Leaf material of a number of varieties was sampled from test fields of the Bundessortenamt in Germany; (2) Another set of varieties was obtained from IPK as in vitro propagated plantlets; (3) The third set was harvested as light sprouts from morphologically verified tubers from the CGN collection (Wageningen, the Netherlands); (4) The fourth set was obtained as DNA from the Laboratory of Plant Breeding, Wageningen University. Varieties used in this study are listed in Appendix 1.

A set of 44 accessions representing 25 tuber-bearing wild and cultivated *Solanum* species plus one outgroup species was used in the analysis (Table 1). Species names followed Hawkes, although we are aware of the synonymy published since then. A selected set of genotypes from the population SHxRH, which has been used for constructing the potato Ultra High Density (UHD) mapping from the Laboratory of Plant Breeding of Wageningen University (Rouppe van der Voort et al. 1998; Huang et al. 2004; Park et al. 2005), was also included. DNA was extracted from leaf

or tuber material using the DNeasy DNA extraction kit (Qiagen).

**Table 1** Species used in the study

| Species | Series[a] | BGRC number |
|---|---|---|
| *S. etuberosum* | ETU | 8082 |
| *S. acaule* ssp. *acaule* | ACA | 28026 |
| *S. acaule* ssp. *acaule* | ACA | 27206 |
| *S. achacachense* | TUB | 29617 |
| *S. berthaultii* | TUB | 28009 |
| *S. berthaultii* | TUB | 24578 |
| *S. bukasovii* | TUB | 15424 |
| *S. bukasovii* | TUB | 18294 |
| *S. bulbocastanum* | BUL | 8009 |
| *S. bulbocastanum* | BUL | 8008 |
| *S. canasense* | TUB | 7162 |
| *S. canasense* | TUB | 8012 |
| *S. chacoense* ssp. *chacoense* | YNG | 17034 |
| *S. chacoense* ssp. *chacoense* | YNG | 17018 |
| *S. coelestipetalum* | TUB | 7993 |
| *S. coelestipetalum* | TUB | 7994 |
| *S. demissum* | DEM | 10030 |
| *S. demissum* | DEM | 10022 |
| *S. gourlayi ssp. gourlayi* | TUB | 7180 |
| *S. gourlayi ssp. Gourlayi* | TUB | 17338 |
| *S. leptophyes* | TUB | 27176 |
| *S. megistacrolobum* ssp. *megistacrolobum* | MEG | 8113 |
| *S. megistacrolobum* ssp. *megistacrolobum* | MEG | 8125 |
| *S. microdontum* | TUB | 31189 |
| *S. microdontum* | TUB | 24649 |
| *S. multidissectum* | TUB | 15426 |
| *S. oplocense* | TUB | 16868 |
| *S. papita* | LON | 15444 |
| *S. phureja* | TUB | 15482 |
| *S. phureja* | TUB | 50199 |
| *S. pinnatisectum* | PIN | 8168 |
| *S. pinnatisectum* | PIN | 8175 |
| *S. raphanifolium* | MEG | 7207 |
| *S. sanctae-rosae* | MEG | 15454 |
| *S. sparsipilum* | TUB | 8150 |
| *S. sparsipilum* | TUB | 8209 |
| *S. stenotomum* ssp. *stenotomum* | TUB | 7478 |
| *S. stoloniferum* | LON | 7229 |
| *S. stoloniferum* | LON | 7228 |
| *S. tuberosum* ssp. *andigena* | TUB | 7462 |
| *S. tuberosum* ssp. *andigena* | TUB | 24677 |
| *S. vernei* | TUB | 15451 |
| *S. vernei* | TUB | 17542 |
| *S. verrucosum* | TUB | 8255 |
| *S. verrucosum* | TUB | 8246 |

[a] Series abbreviations according to Hawkes (1990)

Phenotypic data for the potato varieties were extracted from http:// www.europotato.org, from variable literature information and from national list

publications from the United Kingdom, Germany and The Netherlands during the last 60 years. Pedigree information for the varieties was retrieved from the online pedigree database of the Laboratory of Plant Breeding of Wageningen University, available at http://www.dpw.wau.nl/pv/query.asp.

### NBS profiling procedure and sequencing of NBS profiling fragments

NBS profiling was performed essentially as described by Malosetti et al. (2007). NBS profiling gels were performed by different persons in a range of four years. In total, three NBS primers NBS2, NBS5a6 and NBS9 (Chapter 2) were used on all the materials. A single enzyme *Mse I* was used for NBS profiling of all 456 varieties (Appendix 1). Extra *MseI*-NBS profiles were run with species (Table 1) and a small set of varieties (Appendix 2), ensuring reliably linking variety markers to species markers. In addition, the number of markers was expanded by using three extra primer enzyme combinations (NBS2, NBS5a6 and NBS9 with *Rsa I*).

For sequence comparisons, bands co-migrating at the same positions in varieties, species and the segregating population were excised from the gel, re-amplified using PCR conditions identical to the first PCR of the NBS profiling protocol. Fragments were directly sequenced using either the adaptor or the NBS primer as a sequencing primer with the BigDye Terminator kit and an ABI 3700 automated sequencer from Applied Biosystems (USA). Sequences were analyzed with the seqman module of the Lasergene software package from DNAstar (Madison, USA). Sequences of poor quality were excluded from further analysis, and the remaining sequences were compared to archived sequences in the NCBI nucleotide databases using BLASTN and BLASTX (Altschul et al. 1997).

### Data analysis

Changes in genetic diversity were analyzed using NTSYS-pc software version 2.10j (Rohlf 1992). A genetic similarity matrix was calculated using the Jaccard coefficient. A PCO plot was generated from this matrix using the ordination module.

## Results

### NBS profiling on a large set of potato varieties

The potato varieties used represent the genetic diversity present in the north-western

part of Europe (The Netherlands, United Kingdom, and Germany) during the last 70-80 years. In total 456 varieties were collected and fingerprinted with three primer-enzyme combinations (NBS2, NBS5a6 and NBS9 in combination with *MseI*). The markers were scored as presence/absence, and are therefore dominant. With NBS9, 45 polymorphic markers were scored. NBS2 yielded 23 polymorphic markers, and NBS5a6 51 polymorphic markers.

**Genetic diversity of varieties**

The varieties were chosen to represent the genetic diversity present in three time periods (pre-1950, 1951-1975 and 1976-2000). Active potato breeding has been practised in north-western Europe for over a century, with introgressions from wild potato species starting in late 1930s, and late 1940s and 1950s. Several important disease resistances were targeted and introgressed from species originating from Central and South America. Changes in resistance gene related genetic diversity were analyzed. Only markers that could be scored in the majority of the varieties were taken into consideration (73 markers), and only varieties that had reliable scores for more than 90% of the markers were included (Fig.1). A genetic distance matrix was calculated with the Jaccard coefficient, and a PCO plot was generated from this matrix. Fig.1A shows a PCO plot visualizing the diversity of varieties grown in each of the three timeframes (up to 1950, early 1970s and late 1990s). To highlight the changes over time in genetic diversity of potato varieties, the extremes for each time frame were connected to form convex hulls (Fig.1B). The areas of the convex hulls indicate that there has been an increase in the genetic diversity linked to resistance genes in potatoes grown in Europe in the past 50 years. When the change in diversity was analyzed per country, the changes were even bigger, 23% of the total number of identified alleles was not present in Dutch varieties before 1950 (32% for German pre-1950 varieties), whereas in Dutch varieties from the 1951-1970 period only 11 of the alleles were missing. In the last period, a mere 9% of the total number of alleles was missing from the Dutch varieties (75 for the German varieties). This illustrates that in each of the three countries, the number of alleles had increased considerably over time. This increase is caused by the introduction of new alleles in all countries (as discussed above, most likely by introgression from their wild tuber-bearing *Solanum* relatives. The data also reflects the internationalization of potato breeding, as several alleles that were unique to one of the three countries in the 1950s were present

in varieties that were on the market in all countries during the 1990s.

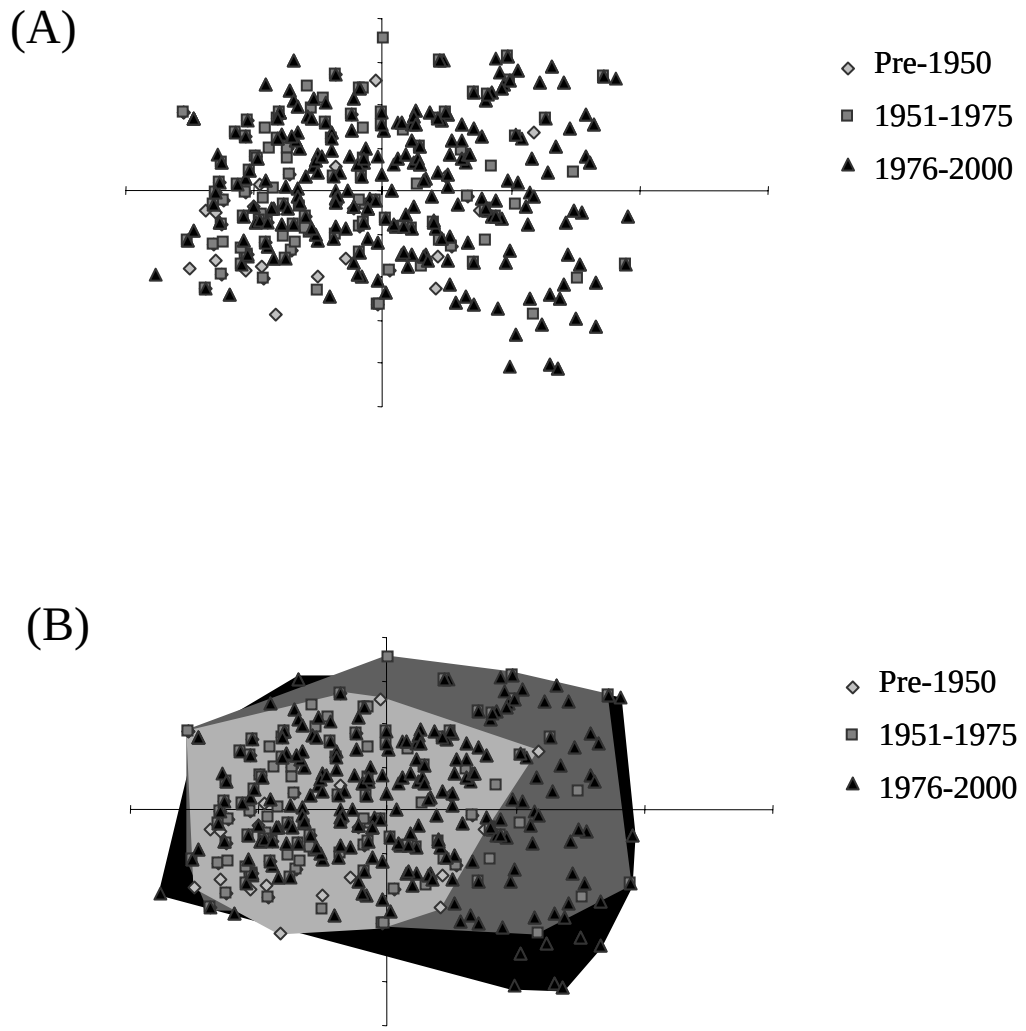


**Fig. 1** Genetic diversity analysis of potato varieties. Seventy three NBS profiling markers were used to assess the genetic diversity in 402 potato varieties. (A) The varieties were categorized relative to their introduction date in three groups: pre-1950, 1951-1975 and 1976-2000. (B) To visualize the changes in genetic diversity over time, convex hulls were drawn for each of the three time periods.

**Marker changes over time**

The presence of each NBS profiling marker in the examined time periods was examined. All markers, except one that was present in the varieties grown before 1950, were still present in those that were grown in 1995-2000, indicating that almost no alleles were lost in the last 60 years. On the contrary, several new markers were introduced into the potato variety gene pool, which may reflect the efforts of breeding companies and research institutes to introgress resistance genes into cultivated potato.

Markers that showed marked increase/decrease over time are summarized in Table 2.

**Table 2** Marked increase/decrease of NBS profiling markers over time

| Marker | Rel. presence pre-1950 (%) | Rel. presence 1951-1975 (%) | Rel. presence 1976-2000 (%) | Sequence homology | Similarity (nt/nt) |
|---|---|---|---|---|---|
| NBS2-03b | 42 | 22 | 18 | No sequence | |
| NBS2-04 | 21 | 53 | 57 | No sequence | |
| NBS2-05 | 61 | 31 | 33 | Tomato Bac clone; new RGA | 166/194 (85%) |
| NBS2-07 | 23 | 57 | 58 | Tomato Bac clone; new RGA | 232/265 (87%) |
| NBS2-07a | 17 | 71 | 57 | No sequence | |
| NBS2-12a | 0 | 4 | 4 | | |
| NBS5-01 | 33 | 11 | 7 | No Sequence | |
| NBS5-06 | 13 | 2 | 7 | Rpi-blb2 (potato) | 503/563 (89%) |
| NBS5-11a | 12 | 0 | 1 | No Sequence | |
| NBS5-12 | 0 | 5 | 19 | No Significant homology | |
| NBS5-15b | 0 | 2 | 7 | No Sequence | |
| NBS5-16 | 0 | 2 | 4 | Potato I2 gene | 250/274 (91%) |
| NBS5-17 | 17 | 33 | 40 | new RGA | |
| NBS5-19 | 81 | 45 | 40 | No significant homology | |
| NBS5-20b | 33 | 28 | 12 | New RGA | |
| NBS5-21 | 0 | 3 | 2 | Tomato Chr IV sequence | 43/53 (81%) |
| NBS5-21c | 7 | 0 | 0 | No sequence | |
| NBS5-22b | 2 | 5 | 8 | I2C-1 (tomato) | 100/110 (90%) |
| NBS5-23 | 2 | 13 | 13 | Potato I2 gene | 157/172 (91%) |
| NBS5-29 | 5 | 8 | 13 | No sequence | |
| NBS9-01 | 4 | 12 | 17 | No Sequence | |
| NBS9-01b | 2 | 6 | 8 | No sequence | |
| NBS9-08 | 0 | 8 | 8 | No Sequence | |
| NBS9-13 | 2 | 10 | 12 | Gpa2/Rx/RGC1 | 234/239 (97%) |
| NBS9-20a | 3 | 9 | 10 | Gpa2/Rx/RGC1 | 119/140 (85% |
| NBS9-31 | 5 | 6 | 11 | Wheat NBS-like sequence; similarity to Gpa2/Rx/RGC1 | 41/42 (97%); sequence short |
| NBS9-31a | 0 | 3 | 5 | No Similarity (Sequence is short) | |

Marker NBS5-12 is not present in any of the varieties that were grown before 1950, and is present in 19% of those that were grown in 1995-2000. This marked increase of NBS5-12 marker presence coincides with breeding efforts to introgress PCN resistance in the 1960s. The earliest introduced variety that carries the NBS5-12 marker is Aladdin, which was introduced in1972. Interestingly, most of the varieties carrying this marker had *Solanum vernei* in their pedigree. *S. vernei* is a donor of *Globodera pallida* resistance, mostly through the breeding line VNT62-33-3 (Ross 1986). In addition, all of the *G. pallida 2/3* resistant varieties in the variety set also carried the marker. Although the data are non-conclusive and for many varieties resistance data are lacking to further support linkage of this marker to PCN resistance, this does suggest that the NBS5-12 marker resides in the introgressed region from *S. vernei* and is physically linked to the *S. vernei* fragment conferring *G. pallida*, and

possibly *G. rostochiensis* resistance (Table 3).

**Table 3** Association between the presence of the candidate *Solanum vernei* marker NBS5-12 and the phenotype. All the varieties have the marker. R=resistant, S=susceptible

| | Phenotype | | | | |
|---|---|---|---|---|---|
| Variety | Ro1 | Ro2, Ro3 | Pa2 | Pa3 | *S. vernei* [a] |
| Albas | - | R | - | - | 1 |
| Arkula | S | - | - | - | 0 |
| Caesar | R | - | - | - | 0 |
| Calgary | R | - | - | - | 1 |
| Calla | R | R | - | - | 1 |
| Darwina | R | R | R | - | 1 |
| Elkana | R | R | - | - | 0 |
| Feska | R | R | R | - | 1 |
| Goya | R | S | R | R | 1 |
| Harmony | R | - | R | R | 1 |
| Hercules | R | - | - | - | 1 |
| Kanjer | R | R | S | - | 1 |
| Karakter | S | R | R | S | 1 |
| Karnico | R | R | R | - | 1 |
| Kartel | R | R | R | R | 1 |
| Katinka | R | R | R | S | 1 |
| Kestrel | R | - | R | R | 0 |
| Krometa | R | R | - | - | 1 |
| Kurola | R | R | R | - | 1 |
| Lady Rosetta | R | - | S | S | 1 |
| Midas | R | - | R | R | 1 |
| Minerva | R | - | - | - | 1 |
| Nadine | R | - | R | R | 1 |
| Nomade | S | R | R | R | 1 |
| Oscar | R | - | - | - | 1 |
| Pallina | R | R | R | R | 1 |
| Proton | R | R | R | - | 1 |
| Santana | R | S | - | - | 1 |
| Santé | R | R | R | - | 1 |
| Seresta | R | R | R | R | 1 |
| Sjamero | R | R | R | R | 1 |
| Spey | R | - | R | R | 0 |
| Vebesta | R | R | R | - | 1 |

[a] When the value is 1, it means that *S. vernei* involves in the pedigree of the variety;
When the value is 0, it means that *S. vernei* does not involve in the pedigree of the variety.

Another interesting marker is NBS9-13, which is present in only one variety prior to 1950 (Industrie), but is much more prevalent in the later time periods. However, no association of the presence of the marker with disease resistance could be found. Markers NBS5-23 and NBS5-16 were also more often present in modern varieties, but again no clear associations with resistances or pedigrees were observed. A few varieties are found in most of the pedigrees of the varieties carrying the NBS5-23 and NBS5-16 markers. These include the varieties Jubel, Pepo and one of

the parents of Industrie, Simson. NBS5-23 was present in the varieties Industrie and Falke (which has the varieties Jubel and Industrie in its pedigree) from the Pre-1950 group. NBS5-16 was not present in pre-1950 varieties.

**Degree of sequence homology among co-migrating NBS profile fragments of different species**

Many markers were identified that migrated at the same positions in wild species, potato varieties (Fig.2) and the individuals from the SHxRH mapping population. In total 76 bands (29 markers) from 24 cultivated and wild *Solanum* species, covering all four clade species (Spooner and Castillo 1997) were excised from both variety and wild species profiles, re-amplified and sequenced, and the sequences compared. In addition, several co-migrating markers were excised and sequenced from profiles of the SHxRH mapping population.

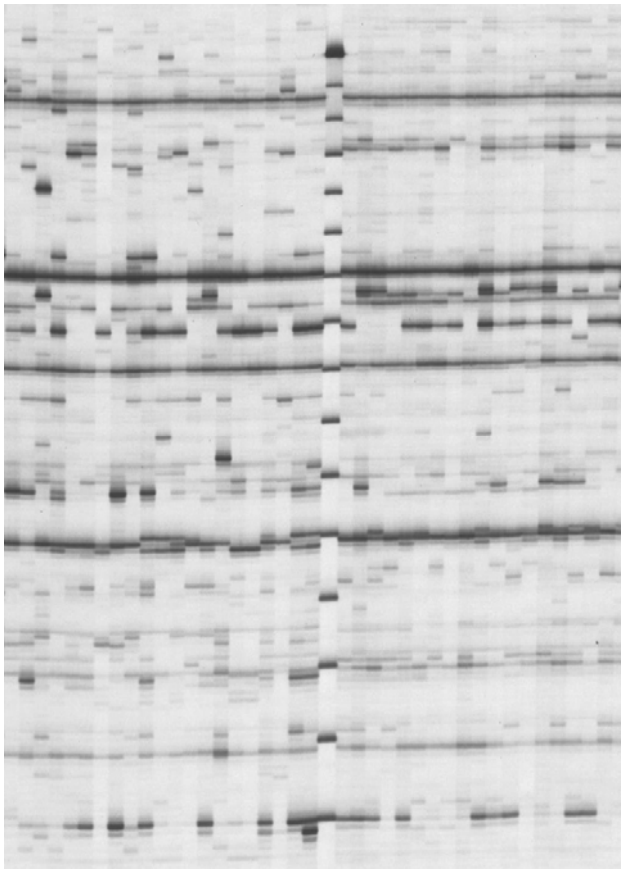

**Fig. 2** An overview of NBS profiling NBS2/Rsa. Left part to the marker is from tuber-bearing *Solanum* species, right part to the marker is from potato varieties.

Six different marker bands were cut both from *Etuberosa* and *Solanum* species.

The sequence homology of three markers between *Solanum* and *Etuberosa* sequences was higher than 95%, indicating that co-segregating markers are most likely originating from the same locus. The sequence of the 4$^{th}$ marker showed 91% homology between the fragments from *Solanum* and *Etuberosa*. The 5$^{th}$ one had indels when compared to corresponding markers in potato species. Excluding the indel, the homology was 95%. The last co-migrating marker was from *Etuberosa* and *S. bulbocastanum*, from which the fragments where 88% homologues to each other.

Considering the tuber-bearing *Solanum* species, potato varieties and the individuals from the segregating population (referred to SHxRH)), interspecific similarities were all higher than 95% for the 64 bands sequenced. In addition there were 6 cases where the homology between bands was lower that 95%, one fragment was 88% homologues to the other fragment, while for the five others, the sequence homology varied between 90% to 95%. Sequence homology among fragments from *Solanum tuberosum*, SHxRH and potato varieties co-migrating markers were all higher than 95% except one, which was 94% homologues to the others.

**Candidate resistance gene markers in varieties, tuber-bearing *Solanum* species and segregating population**

To gain insight in the origin of the NBS profiling markers that are present in cultivated potato and more specifically of the markers that may have been introgressed during the last 50-60 years, we have compared NBS profiles of varieties with profiles of a broad set of wild tuber-bearing *Solanum* species. For this, extra NBS profiles were produced with wild species as well as a set of varieties with diverse pedigrees on the same gel to be able to link as many variety markers as possible to markers in wild species. In addition, the number of markers was expanded by using three extra primer enzyme combinations (NBS2, NBS5a6 and NBS9 with Rsa I).

The NBS5-12 marker was present in two *S. vernei* accessions examined. This is extra support for this marker to be located in the region from *S. vernei* that was introgressed to cultivated potato for nematode resistance. In addition, the marker could also be identified in the two *S. microdontum* accessions examined. The marker was sequenced and compared to the nucleotide databases. No significant similarity was found with BlastX and Megablast searches.

Marker NBS9-13 was found to be present in the majority of the wild species,

and could therefore not be traced to a specific donor species. The marker was sequenced, and found to be 97% identical to the *RGC1* gene residing in the *Gpa2* cluster (Bakker et al. 2003).

Marker NBS5-23 was present in *Solanum stoloniferum* and in *Solanum demissum*. The marker sequence was highly similar to I2C genes from tomato and genes from the R3 complex locus of potato conferring *Phytophthora infestans* resistance (Huang et al. 2005). This marker was not present in the SHxRH mapping population.

Blast analysis showed that some fragments were highly homologous to cloned potato resistance genes, such as R3a and R1. Marker R206-41-v (236bp) from the variety 'Shepody' was annotated as the potato late blight resistance protein R3a (blastx E value=2e-20). The same marker was also present in the segregating population and located on chromosome 11 position RH11B82-83 (results not shown). The sequence from the fragment segregating in the SHxRH population was exactly identical to the sequence of the 'Shepody' fragment.

## Discussion

We have assessed changes in genetic diversity in the cultivated gene pool of potato during the last century, using NBS profiling. Our results show that between 1950 and 1975, the R-gene pool in cultivated potato has slightly widened, which makes sense, as during the last 60-70 years potato breeding has been strongly focused on introgression of R-genes from wild species into the cultivated potato gene pool. The specific advantage of using NBS profiling in this study is illustrated by the fact that when using "neutral" markers (SSRs, and a collection of SNPs), the genetic diversity appears to be unchanged (Reeves et al, 2005).

### Homoplasy in co-migrating bands is low

Taking advantage of the diverse set of materials included in this study, the allelic nature of NBS profiling fragments was assessed by directly sequencing co-migrating markers across a wild range of *Solanum* species, together with potato varieties and the segregating population. This is an important issue not only for systematic studies but also for this study as we like to trace back introgressed regions to the donating wild

species. For this it is essential that one can be sure that co-migrating fragments are indeed similar. To our knowledge, the homoplasy issue has not been addressed for NBS profiling.

Within tuber-bearing *Solanum* species (excluding the *Etuberosum* accession which is not potato, but often used as outgroup for potato phylogenetic studies), the minimum interspecific identity found was 88%, indicating that some related co-migrating markers might be paralogues rather than orthologs (allelic fragments). Our samples cover four clades of tuber-bearing *Solanum* species, which are genetically very diverse (Spooner and Castillo 1997). In total, within tuber-bearing *Solanum* species, 91% of the sequences (58/64) for the same band are more than 95% homologous to each other. Homoplasy in amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) has been studied in the past. In a wild sunflower species complex, 220 co-migrating RAPD fragments were analyzed. Using Southern hybridization and digestion of fragments, it was shown that only 79.1% were homologous (Rieseberg 1996). Homoplasy among AFLP fragments was studied in *Hordeum*, where bands that were identical across species in both signal intensity and migration position were included. The interspecific homology between bands varied significantly, some of them were lower than 40% homologues, others ranged from 82 to 100%. It was suggested to carefully select AFLP bands for systematic study (El-Rabey et al. 2002).

**Markers traits association**

We have identified the marker NBS5-12 as a putative marker linked to a PCN resistance locus that was introgressed in varieties in the 1960s. Both available resistance data and pedigree information indicated that this marker may be introgressed from *S. vernei*. This marker was also present in *S. vernei* accessions, which complies with a possible *S. vernei* origin of the marker. The NBS5-12 marker is not present in accessions of other wild species except the *Solanum microdontum* accessions. Systematically, *S. vernei* and *S. microdontum* belong to a group of species in series *Tuberosa*, occurring in Bolivia and Argentina. The presence of this marker in both species may indicate a common ancestry of this marker. Bryan et al. (2002) have studied *G. pallida* resistance introgressed from *S. vernei* at the tetraploid level, and identified two major QTLs on Chromosomes V and IX. They produced a PCR marker specific for the resistance locus, although their marker was present only in two of the

three varieties with presumed *S. vernei*-introgressed PCN resistance (Santé and Spey were positive, Nadine was negative). The NBS5-12 marker is present in Santé, Spey and Nadine, indicating that it may be tightly linked to the *S. vernei*-introgressed PCN resistance in these varieties. However, the sequence of the marker does not reveal any information pointing to a specific gene or locus: The sequence is not significantly similar to any entry in the public nucleotide databases, and we could not identify any specific motifs/elements indicating that this marker is part of a resistance gene. The sequence is 93% similar for 120 nt (the marker itself is 269nt) to a cDNA from a late blight-challenged library. This cDNA (Genebank DN590692) is as yet not functionally annotated, and is most likely not a member of the NBS-LRR gene family.

Several other markers were identified that either were introduced into the cultivated potato gene pool or considerably increased in frequency over time. However, no clear associations with resistance data could be found for those markers. This may be due to limited resistance data available. Alternatively, resistance data was assembled over a number of years from different sources, scored under varying environmental conditions and possibly using different inoculates, which all would decrease the reliability of the data and the chance of finding a link between markers and resistance. In addition, introgressed R genes might be broken down, rendering the variety susceptible, which will make it more difficult to trace the introgression, because of unreliable resistance data after this.

The NBS5-16 and NBS5-23 markers appear to have been selected in the last 30-40 years in the cultivated gene pool. Sequences of both markers are highly similar to the I2C genes and R3 (Huang et al. 2005) located on Chromosome XI. The R3 locus appears to be a hot spot for disease resistance, harbouring resistance genes against late bight, potato virus A and Y (Huang et al. 2005, Hämäläinen et al. 1997). However, the high similarity does not imply that NB5-16 and NBS5-23 are markers for this locus. As was shown earlier in tomato (Pan et al. 2000), I2C homologs are located at multiple chromosomes, and we have identified a number of NBS profiling markers with I2C similarity at different chromosomal locations (unpublished results). Within the marker set used for the diversity analysis of potato varieties, several other markers with high similarity to I2C were found.

An alternative approach for finding resistance markers in a set of varieties is association mapping, as reported by Malosetti et al. (2007). Using a subset of the varieties presented in this chapter and the same NBS profiling marker data, two

markers were identified with significant association to late blight resistance using a mixed model approach. It would be interesting to see the distribution of these markers over the wild accessions. Unfortunately, the markers from this study could not be scored reliably in the wild accessions. One of the markers was mapped in SHxRH and found to be present in the region harboring the R3 complex on Chromosome XI, which complies with an association of this marker with late blight resistance.

Previously, marker-trait associations were successfully used to assess the genetic potential of the potato germplasm collections (Gebhardt et al. 2004). In their study, a collection of 600 varieties and some wild species were used. DNA markers linked to previously mapped QTLs for resistance to late blight and plant maturity were evaluated. A highly significant association with QTL for resistance to late blight and plant maturity was detected with R1-specific PCR marker. In addition, this marker was traced to an introgression from the wild species *S. demissum*. Molecular and late blight phenotype data showed that the R1 marker was also present in another wild species *S. stoloniferum* (Gebhardt et al. 2004), indicating that R1 containing materials cannot with a 100% certainty be traced back to *S. demissum*.

Our study showed that genetic diversity reflected by NBS profiling in European potato varieties did not decrease during the last 70–80 years. On the contrary, several new markers were introduced into the potato variety gene pool, which may reflect the breeding efforts to introgress resistances from wild species into cultivated potato. *Solanum vernei* marker suggests that NBS profiling can be used to identify candidate R gene markers. Low homoplasy (91% sequences have homology higher than 95%) indicates that NBS profiling can be used for systematic studies within tuber-bearing *Solanum* species.

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# Chapter 4

# Allele mining in *Solanum:* conserved homologues of *Rpi-blb1* are identified in *Solanum stoloniferum*

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**Abstract**

Allele mining facilitates the discovery of novel resistance (R) genes that can be used in breeding programs and sheds light on the evolution of R genes. Here we focus on two R genes, *Rpi-blb1* and *Rpi-blb2*, originally derived from *Solanum bulbocastanum*. The *Rpi-blb1* gene is part of a cluster of four paralogues and is flanked by *RGA1-blb* and *RGA3-blb*. Highly conserved *RGA1-blb* homologues were discovered in all the tested tuber-bearing (TB) and non-tuber-bearing (NTB) *Solanum* species, suggesting *RGA1-blb* was present before the divergence of TB and NTB *Solanum* species. The frequency of the *RGA3-blb* gene was much lower. Interestingly, highly conserved *Rpi-blb1* homologues were discovered not only in *S. bulbocastanum* but also in *Solanum stoloniferum* which is part of the series *Longipedicellata*. Resistance assays and genetic analyses in several F1 populations derived from the relevant late blight resistant parental genotypes harbouring the conserved *Rpi-blb1* homologues, indicated the presence of four dominant R genes, designated as *Rpi-sto1*, *Rpi-plt1*, *Rpi-pta1* and *Rpi-pta2*. Furthermore, we show that *Rpi-sto1* and *Rpi-plt1* reside at the same position on chromosome VIII as *Rpi-blb1* in *S. bulbocastanum*. Segregation data also indicated that an additional unknown late blight gene was present in three populations. In contrast to *Rpi-blb1*, no homologues of *Rpi-blb2* were detected in any other material examined. Hypotheses are proposed to explain the presence of conserved *Rpi-blb1* homologues in *S. stoloniferum*. The discovery of conserved homologues of *Rpi-blb1* in

tetraploid species offers the possibility to more easily transfer the late blight resistance genes to potato cultivars by classical breeding.

## Introduction

Late blight is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary and it is one of the most important diseases affecting the potato crop *Solanum tuberosum* L. worldwide. The management of the disease has been estimated to cost $ 3.5 billion annually (GILB 2004). To reduce the cost of the disease and the environmental damage, it is important to identify resistance that can be used in breeding programs. In the past, 11 major resistance genes (R-genes) were introgressed from hexaploid *S. demissum* into cultivated potato (Black et al. 1953; Malcolmson and Black. 1966) and all these genes confer race-specific resistance. Unfortunately, the resistance based on these genes was quickly overcome by the pathogen. Hence, new sources of resistance are required to develop late blight resistant potato varieties.

More late blight R-genes have been found and mapped in wild diploid species. Examples are *S. pinnatisectum* (Kuhl et al. 2001) and *S. bulbocastanum* (Naess et al. 2000; Song et al. 2003; Van der Vossen et al. 2003 and 2005; Park et al. 2005) from Mexico and *S. berthaultii* (Ewing et al. 2000), *S. microdontum* (Sandbrink et al. 2000), *S. mochiquense* (Smilde et al. 2005) and *S. paucissectum* (Villamon et al. 2005) from Andean countries. In addition, the cultivated diploid *S. phureja* has been described to contain valuable resistance (Ghislain et al. 2001; Sliwka et al. 2006). The presence of late blight R-genes in the above mentioned species indicates that wild and so called primitive germplasm is a rich source for novel R-genes that may be exploited in breeding programs.

To date, four late blight R-genes have been cloned: *R1* (Ballvora et al. 2002), *R3a* (Huang et al. 2005), *RB* or *Rpi-blb1* (Song et al. 2003; van der Vossen et al. 2003) and *Rpi-blb2* (van der Vossen et al. 2005). The latter two genes were cloned from *S. bulbocastanum* and confer resistance to all *P. infestans* isolates tested so far. *Rpi-blb1* is part of a resistance gene analog (RGA) cluster of four members *RGA1-blb*, *Rpi-blb1*, *RGA3-blb*, and *RGA4-blb* on chromosome VIII (van der Vossen et al. 2003). Complementation analysis showed that only the genetic construct harbouring *Rpi-blb1* was able to complement the susceptible phenotype. The sequence relationships between *Rpi-blb1*, *RGA1-blb* and *RGA3-blb* showed that the *Rpi-blb1* gene most likely evolved from intragenic recombination between the ancestral genes of *RGA3-blb* and *RGA1-blb* (van der Vossen et al. 2003). *Rpi-blb2* resides in a locus harbouring

at least 15 *Mi-1* gene homologues on chromosome VI, and the *Rpi-blb2* protein shows 82% sequence identity to the *Mi-1* protein (van der Vossen et al. 2005). *Mi-1* is a gene of tomato that confers resistance to the root knot nematode *Meloidogyne incognita* (Milligan et al. 1998).

This study aims at analysing the allele frequency and allelic variation of *Rpi-blb1* and *Rpi-blb2* in a large number of tuber-bearing *Solanum* species and it explores the genomic organization of the *Rpi-blb1* cluster in these species. Insight into allelic diversity will facilitate discovery of functional homologues that can be exploited in breeding programs and will also help to understand the evolution of R-genes.

## Materials and methods

### Plant material and DNA extraction

Material was selected to represent most series of the section *Petota*, based on their systematic relationships derived from AFLP and NBS profiling data (Chapter 2). Seeds were obtained from several genebanks (Table 1). Individual clones were surface-sterilized and sown in vitro for at least 6 weeks on MS medium supplemented with 20% sucrose (Murashige and Skoog, 1962) at 18$^{o}$C. In total, 86 genotypes covering 47 species representing 13 series (three genotypes from series *Etubersosa*) were used (Table 1). DNA was extracted according to the method described by Stewart and Via (1993). Species names and abbreviations follow Hawkes (1990), since the genebank material used is labeled as such. However, we refer to *S. stoloniferum* (sensu Spooner et al. 2004) to indicate their broader species concept, which considers *S. fendleri, S. papita* and *S. polytrichon* as synonyms of *S. stoloniferum*.

**Table 1** Material used for the identification of *Rpi-blb1* cluster and *Rpi-blb2* homologues and amplification of *Rpi-blb1* cluster members

| Species[a] | Genebank[b] | BLB1F/R | RGA1F/R | RGA3F/R |
|---|---|---|---|---|
| *S. etuberosum* | 18242 | 0 | 1 | 0 |
| *S. fernandezianum* | 18360 | 0 | 1 | 0 |
| *S. palustre* | 18241 | 0 | 1 | 0 |
| *S. acaule* ssp. *acaule* | BGRC7949 | 0 | 1 | 0 |
| *S. acaule* ssp. *aemulans* | 21331 | 0 | 1 | 0 |
| *S. ajanhuiri* | 18239 | 0 | 1 | 1 |
| *S. berthaultii* | 20644, 20650 | 0 | 1 | 1 |
| *S. brachistotrichium* | 17681 | 0 | 1 | 0 |
| *S. brachycarpum* | 17721(3),18347, CPC7028, GLKS1686 | 0 | 1 | 0 |
| *S. brevicaule* | 18231 | 0 | 1 | 0 |
| *S. bukasovii* | 17824 | 0 | 1 | 0 |
| *S. bulbocastanum* | 17687 | 1 | 1 | 1 |
| *S. bulbocastanum* | 17691 | 0 | 1 | 1 |
| *S. canasense* | 17589 | 0 | 1 | 0 |
| *S. cardiophyllum* | 18326 | 0 | 1 | 1 |
| *S. chacoense* | 18248-1, 18248-4 | 0 | 1 | 1 |
| *S. chacoense* | 18248-9 | 0 | 1 | 0 |
| *S. circaeifolium* | 18133 | 0 | 1 | 0 |
| *S. circaeifolium* ssp. *quimense* | 18127 | 0 | 1 | 0 |
| *S. demissum* | 20571 | 0 | 1 | 0 |
| *S. fendleri* ssp. *arizonicum* | PI497996 | 0 | 1 | 0 |
| *S. guerreroense* | 18290(2), GLKS1512 | 0 | 1 | 0 |
| *S. hjertingii* | 18345 | 0 | 1 | 0 |
| *S. hondelmannii* | 18106, 18182(2) | 0 | 1 | 0 |
| *S. hougasii* | 18339(2) | 0 | 1 | 0 |
| *S. huancabambense* | 17719 | 0 | 1 | 0 |
| *S. iopetalum* | 20561 | 0 | 1 | 1 |
| *S. iopetalum* | 20562 | 0 | 1 | 0 |
| *S. jamesii* | 18349 | 0 | 1 | 0 |
| *S. leptophyes* | 18140 | 0 | 1 | 0 |
| *S. lesteri* | 18337 | 0 | 1 | 1 |
| *S. megistacrolobum* | GLKS5422 | 0 | 1 | 0 |
| *S. microdontum* | 17596 | 0 | 1 | 1 |
| *S. microdontum* ssp. *gigantophyllum* | 18046, 18200 | 0 | 1 | 0 |
| *S. mochiquense* | 18263(2) | 0 | 1 | 0 |
| *S. oxycarpum* | 20558 | 0 | 1 | 0 |
| *S. papita* | 17831 | 1 | 1 | 1 |
| *S. paucissectum* | PI590922 | 0 | 1 | 0 |
| *S. phureja* | 18301 | 0 | 1 | 0 |
| *S. pinnatisectum* | 17745(3), 23012 | 0 | 1 | 1 |
| *S. polyadenium* | 17749 | 0 | 1 | 1 |
| *S. polytrichon* | 22361 | 0 | 1 | 0 |
| *S. raphanifolium* | 17753(2) | 0 | 1 | 0 |
| *S. sanctae-rosae* | 17837, 20576 | 0 | 1 | 0 |
| *S. schenckii* | 18361 | 0 | 1 | 1 |
| *S. sparsipilum* | 18221 | 0 | 1 | 0 |
| *S. sparsipilum* | 18225 | 0 | 1 | 1 |

**Table 1** Continued

| Species[a] | Genebank[b] | BLB1F/R | RGA1F/R | RGA3F/R |
|---|---|---|---|---|
| *S. stoloniferum* | 17605, 17606, BGRC60465, CPC28 | 1 | 1 | 1 |
| *S. stoloniferum* | 18333, GLKS592 | 0 | 1 | 1 |
| *S. stoloniferum* | 17607, 18332, 18334, 18348, 23072, CPC12, GLKS512 | 0 | 1 | 0 |
| *S. sucrense* | 18205 | 0 | 1 | 0 |
| *S. tarijense* | 17861 | 0 | 1 | 1 |
| *S. tuberosum* ssp. *andigena* | 20614 | 0 | 1 | 1 |
| *S. vernei* | 21350 | 0 | 1 | 1 |
| *S. verrucosum* | 20567(2) | 0 | 1 | 0 |

[a]Materials are ordered alphabetically with the exception that three genotypes from series *Etuberosa* are listed first as they are non-tuber-bearing *Solanum* species.

[b]Materials starting with a number directly are from Center for Genetic Resources, the Netherlands (CGN). Materials starting with BGRC, CPC, GLKS and PI are from Braunschweig Genetic Resources Collection, the Commonwealth Potato Collection (Dundee, Scotland), Gross Lusewitz, Konrad Schuler (Germany), United States Department of Agriculture Plant Introduction Numbers, respectively. Numbers between parenthesis refer to the number of the genotype within one accession. Genotype number is provided only when PCR patterns within/among the genotypes differ.

**R-gene specific primers**

All *Rpi-blb1* primers designed in this study (Table 2) were tested on the clone 8005-8 (BGRC accession number 8005, individual plant 8) from which the *Rpi-blb1* gene and its paralogues *RGA1-blb* and *RGA3-blb* were cloned (van der Vossen et al. 2005). *Rpi-blb2* primers (Table 2) were tested on the late blight resistant clone Blb2002, the diploid *S. bulbocastanum* clone from which *Rpi-blb2* was cloned (van der Vossen et al. 2005).

**Segregating populations and resistance assays**

In case of *S. stoloniferum*, a late blight resistant clone CGN17605-4, was crossed with the susceptible breeding clone RH89-039-16, which produces 2n pollen and is frequently used for mapping research at the Laboratory of Plant Breeding of Wageningen University and Research Centre (Rouppe van der Voort et al. 1998; Huang et al. 2004; Park et al. 2005). For the late blight resistance test, detached leaf assays (DLAs) were performed as described by Vleeshouwers et al. (1999). The *P. infestans* isolates IPO82001, 655-2A (Flier et al. 2003) were from the collection of PRI, isolate “Marknesse” was from a diseased potato collected in 2005 near the Dutch village Marknesse.

**Table 2** Primers overview

| Primer | F/R[a] | Sequence (5'-3')[b] | Anealing temperature (°C) | length of 72 °C extention | Reference |
|---|---|---|---|---|---|
| BLB1F/R | F | AACCTGTATGGCAGTGGCATG | 58 | 50" | |
| | R | GTCAGAAAAGGGCACTCGTG | | | |
| 517/1519 | F | CATTCCAACTAGCCATCTTGG | 58 | 50" | |
| | R | TATTCAGATCGAAAGTACAACG | | | |
| 1521/518 | F | GAAAGTCTAGAGTTACACTGG | 58 | 50" | |
| | R | CAATCACAATGGCAGGAACC | | | |
| BLB1F/GR | F | AACCTGTATGGCAGTGGCATG | 55 | 1'40" | |
| | R | GYTGTTAGGTGCTGCAATCC | | | |
| BLB1GF/R | F | GWGMATGGGAACATGTGAGAG | 55 | 1'40" | |
| | R | GTCAGAAAAGGGCACTCGTG | | | |
| RGA1F/R | F | CAGTCACTTTCTTGTTTGCCG | 55 | 55" | |
| | F | CAGTAGTGAAGTCACTGTGTG | | | |
| RGA3F/R | F | CATGCCTTAAGTCTCTAAGTTG | 55 | 55" | |
| | R | TGGGAGTGAAGTAGCTTCTAC | | | |
| BLB2F/R | F | GGACTGGGTAACGACAATCC | 58 | 50" | |
| | R | GCATTAGGGGAACTCGTGCT | | | |
| BLB2F/GR | F | GGACTGGGTAACGACAATCC | 55 | 1'40" | |
| | R | ATTTATGGCTGCAGAGGACC | | | |
| BLB2GF/R | F | ATTGCTGGARTCATTGCTGG | 55 | 1'40" | |
| | R | GCATTAGGGGAACTCGTGCT | | | |
| 1+1' | F | CACGAGTGCCCTTTTCTGAC | 50 | 2' | Colton et al. 2006 |
| | R | ACAATTGAATTTTTAGACTT | | | |
| CT88 | F | GGCAGAAGAGCTAGGAAGAG | 60 | 50" | van der Vossen et al. 2003 |
| | R | ATGGCGTGATACAATCCGAG | | | |

[a] F: forward primer; R: reverse primer

[b] Nucleotide abbreviations according to the IUB code (M, AC; R, AG; W, AT; Y, CT )

Three other populations were constructed using plant material that is now known as *S. stoloniferum* (sensu Spooner et al. 2004). However, in the genebank catalog these materials are still present under their previous names *S. papita* and *S. polytrichon*. Two tetraploid mapping populations for *S. papita* (Pta), Pta 04-323 and Pta 04-325, were obtained by backcrossing two resistant offspring, Pta 03-390-1 and Pta 03-390-3 respectively, with a susceptible *S. polytrichon* (Plt) pollen donor from accession CGN 17751. Pta 03-390-1 and Pta 03-390-3 were both derived from a cross between a susceptible plant from Pta accession CGN 18319 and a resistant plant from Pta accession CGN 17831. In 2004, 48 plants from this cross, which included Pta 03-390-1 and Pta 03-390-3, were found to be completely resistant in a single DLA with *P. infestans* isolate IPO82001. Similarly, a segregating population of Plt was obtained (Plt 04-281) by crossing the resistant clone Plt 03-369-1 with a susceptible clone from Pta accession CGN18319. Plt 03-369-1 was derived from a cross between a susceptible plant from Pta accession CGN 18319 and a resistant plant from Plt accession CGN 18318.

Again, this cross showed a fully resistant offspring after screening 72 plants in a DLA with the IPO82001 isolate.

**PCR amplification**

Three pairs of primers (BLB1F/R, 517/1519, 1521/518, Table 2) were designed based on the *Rpi-blb1* homologous sequences (van der Vossen et al. 2003), aiming at specific amplification of the *Rpi-blb1* gene. Two degenerate primers (upstream and downstream of the primer BLB1F/R) were designed to determine whether non-amplification with the BLB1F/R primer pair might be caused by a mutation in one of the primer site. In addition, primers for the *Rpi-blb1* paralogues *RGA1-blb* and *RGA3-blb* were developed. A similar approach was followed for *Rpi-blb2*, again by designing specific and degenerate primers. Figure 1 shows the locations for the *Rpi-blb1* and *Rpi-blb2* primers designed in this study. PCR reactions were carried out in a 15µl reaction system, containing approximately 100ng DNA, 2.25 pmol of each primer, 3 mM of each dNTP, 0.6 units Taq-poplymerase (15 U $\mu l^{-1}$, SphaeroQ, Leiden, the Netherlands), 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 0.01% (w/v) gelatine. The PCR protocol started with 5 min at 95°C. The 35-cycle amplification profiles were as followed: 30-sec DNA denaturation at 94°C, 40-sec annealing and variable elongation (depending on the primer, Table 2) at 72°C. The PCR was finalized by an extra 5 min elongation step at 72°C. The PCR protocol used for primer 1 + 1' was according to Colton et al. (2006).

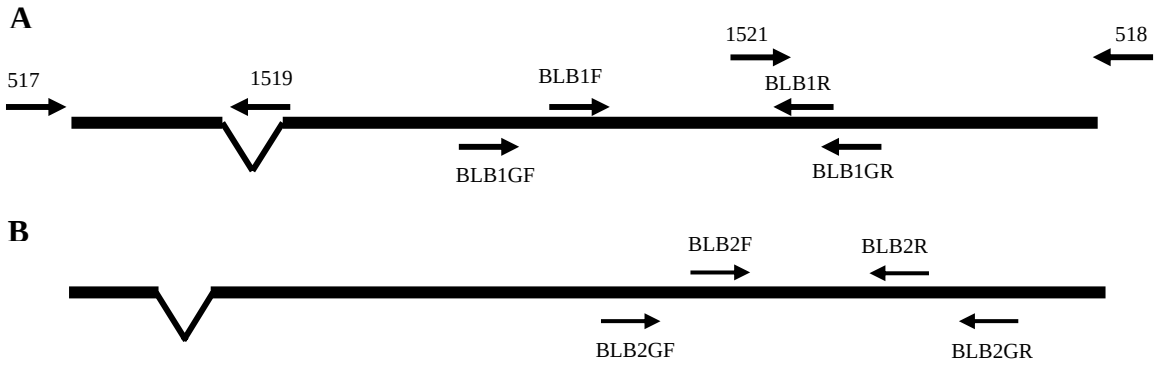


**Fig. 1** Locations of the primers designed in this study, drawing not to scale

(**A**) *Rpi-blb1* related primers. Forward primer 517 from the primerpair 517/1519 is located upstream of the start codon of Rpi-blb1, while reverse primer 518 from the primerpair 1521/518 is located downstream of the stop codon. Primer BLB1GF and BLB1GR is located upstream and downstream of the primer BLB1F/R. Reverse primer 1519 is located in the intron of *Rpi-blb1*, while all other primers (except 517 and 1521) are located in the exon of *Rpi-blb1*. (**B**) *Rpi-blb2* related primers

The chromosome VIII specific marker CT88 (van der Vossen et al. 2003) was initially tested on the parents of all four segregation populations. Following digestion of the PCR products with specific restriction enzymes, polymorphic markers were subsequently tested in the entire population. All amplification reactions were performed in a Biometra® T-Gradient or Biometra® Uno-II thermocycler (Westburg, Leusden, the Netherlands). PCR products were separated in 1.5% agarose gels and stained with ethidiumbromide.

**Data collection and sequence analysis**

For selected genotypes, PCR products were sequenced directly to confirm their identity and to identify single nucleotide polymorphisms (SNPs). Each fragment was sequenced from both sides using the two primers as a sequencing primer with the BigDye Terminator kit. Sequencing reactions were analysed using an ABI 3700 automated sequencer (Applied Biosystems, USA). DNA sequences were analysed using DNAstar (Lasergene, Madison, WI, USA).

## Results

**Primer specificity**

All *Rpi-blb1* and *Rpi-blb2* related primers designed in this study (Table 2) were tested on the clone Blb 8005-8), from which *Rpi-blb1* and its paralogues *RGA1-blb* and *RGA3-blb* were cloned, or on clone Blb2002, which was used for the cloning of *Rpi-blb2* (van der Vossen et al. 2005). Sequence analysis showed that all primers amplified the expected fragments with two exceptions: (1) poor sequences for the BLB1GF/R primer product were obtained, suggesting that this primer pair amplified a mixture of related sequences; (2) compared with AY426261 (*RGA3-blb*), double peaks were found at five nucleotide positions. Except for these five SNPs, the remainder of the sequence was identical to AY426261.

***RGA1-blb* and *RGA3-blb* homologues**

With the RGA1F/R specific primers, *RGA1-blb* homologues were amplified not only from all the tuber-bearing *Solanum* genotypes but also from three non-tuber-bearing *Solanum* genotypes *S. etuberosum, S. fernandezianum* and *S. palustre* (Table 1). RGA1F/R derived sequences from 36 randomly selected genotypes were highly homologous (96%-99%) to that of *RGA1-blb* (results not shown). In contrast, a much smaller set of genotypes contained

RGA3 homologues (Table 1). RGA3F/R derived sequences from different genotypes were highly homologous to *RGA3-blb* AY426261(88%-98%).

### *Rpi-blb1* in *Solanum* species

Screening of more than 80 genotypes (Table 1) with three pairs of primers, primer pair BLB1F/R and the two degenerate primer pairs BLB1F/GR and BLB1GF/R (Table 2), showed that the primers BLB1F/R amplified fragments from genotypes in *S. bulbocastanum* and *S. stoloniferum* (sensu Spooner et al. 2004). In these genotypes, primer pairs BLB1F/GR and BLB1GF/R also amplified fragments. Combined sequences from BLB1F/R and BLB1F/GR derived fragments showed that these fragments were highly homologous to that of *Rpi-blb1*. BLB1GF/R amplicons from some genotypes contained the 18 basepair sequence that is characteristic for the *Rpi-blb1* resistance allele (Song et al. 2003), while others did not. This indicated that the BLB1GF/R primer amplified both the R and S allele. In some BLB1GF/R products heterogeneity was observed, indicating that more than one homologue was amplified. All genotypes that contained the *Rpi-blb1* specific allele also contained conserved *RGA1* and *RGA3* homologues.

In some genotypes, primer set BLB1F/R did not amplify fragments, while the degenerate primer set BLB1F/GR did. These genotypes can be classified into three groups: genotypes showing, 1) one single fragment of the expected size, 2) one single fragment of smaller size (data not shown), and 3) one single fragment of the expected size and the other of smaller size (data not shown). Sequences from the first group did not contain the reverse primer BLB1R, which explains why BLB1F/R primers did not amplify fragments. For this reason, these genotypes were excluded in further analyses.

After identifying the *Rpi-blb1* homologues, more accessions and genotypes (Table 3) of the series *Longipedicellata* (Hawkes 1990) were screened for the presence of *Rpi-blb1* homologues with the primers BLB1F/R, 517/1519 and 1521/518. As expected, more accessions and genotypes were found to contain highly conserved *Rpi-blb1* homologues (Table 3), which also harboured the 18bp-sequence that is characteristic for the functional *Rpi-blb1* gene (Song et al. 2003). Based on the obtained partial sequences, three haplotypes were discovered (Table 4). For haplotype 1 and 3, SNPs at positions 64 and 65 together changed the amino acid from Val to Thr, while in haplotype 2, Val was changed to Ala. Another four SNPs at positions 2664, 3134, 3255 and 3588 changed the amino acid, from Lys to Arg, Met to Leu, Ala to Glu and Ile to Asn, respectively.

**Table 3** Extended materials used to identify the *Rpi-blb1* and *Rpi-blb2* homologues

| | | Primers for *Rpi-blb1* | | |
|---|---|---|---|---|
| Species | Genebank[a] | BLB1F/R | 517/1519 | 1521/518 |
| *S. papita* | 17830(3), 18309(2), 18319(2) | 0 | 0 | 0 |
| *S. papita* | 17831(4) | 1 | 1 | 1 |
| *S. papita* | 17832-1, 17832-5 | 0 | 0 | 0 |
| *S. papita* | 17832-2 | 1 | 1 | 1 |
| *S. polytrichon* | 18318-(1 to 4), 18318-(6 to 9) | 0 | 0 | 0 |
| *S. polytrichon* | 18318 | 1 | 1 | 1 |
| *S. stoloniferum* | 17606, 17607, 18332, 18333, 18348(2), CPC12, GLKS512 | 0 | 0 | 0 |
| *S. stoloniferum* | 18334-1 | 0 | 0 | 0 |
| *S. stoloniferum* | 18334-8 | 0 | 0 | 1 |
| *S. stoloniferum* | 17605(4), BGRC60465-3 | 1 | 1 | 1 |

[a]Materials starting with a number directly are from Center for Genetic Resources, the Netherlands (CGN). Materials starting with BGRC, CPC and GLKS are from Braunschweig Genetic Resources Collection, the Commonwealth Potato Collection (Dundee, Scotland) and Gross Lusewitz, Konrad Schuler (Germany), respectively. Numbers in parenthesis refer to the number of the genotypes included in that one accession. Genotype number is provided only when PCR patterns within/among the genotypes differ.

**Table 4** Nucleotide polymorphisms of *Rpi-blb1* homologues from three pairs of primers BLB1F/R, 517/1519 and 1521/518. Rpi-blb1 sequences are as reference for comparison. Grey background positions mean that the SNP changes the amino acid.

| | | Sequences amplified from three pairs of primers[a] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | Materials[b] | 28 | 64 | 65 | 123 | 210 | 315 | 266 | 313 | 325 | 358 | Number of SNP | Haplotype |
| | Rpi-blb1 | C | G | T | T | T | C | A | A | C | T | | |
| *S.. papita* | 17831-1 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S.. papita* | PTA 03-390-1 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S.. papita* | PTA 03-390-3 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S.. papita* | 17831-8 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S.. papita* | 17832-2 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S. polytrichon* | PLT 03-369-1 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S. stoloniferum* | BGRC60465-3 | | | C | | | T | | | A | | 3 | 2 |
| *S. stoloniferum* | 17605-1 | T | A | C | C | C | | | | | A | 6 | 3 |
| *S. stoloniferum* | 17605-2 | T | A | C | C | C | | | | | A | 6 | 3 |
| *S. stoloniferum* | 17605-3 | T | A | C | C | C | | | | | A | 6 | 3 |
| *S. stoloniferum* | 17605-4 | T | A | C | C | C | | | | | A | 6 | 3 |

[a] Sequences from start codon till position 542 are from the primer 517/1519 and sequences from 1737 till stop codon are from the combination of the primer BLB1F/R and 1521/518.

[b] Materials starting with a number directly are from Center for Genetic resources in the Netherlands (CGN).Materials starting with BGRC are from Braunschweig Genetic Resources Collection.Genotype information is given after the genebank number. Detail for three genotypes of the segregating populations PTA 03-390-1, PTA 03-390-3, PLT 03-369-1, see Materials and Methods.

**Identification and mapping of the genes *Rpi-sto1, Rpi-plt1, Rpi-pta1 and Rpi-pta2***

Progenies (33 individuals) of a cross between the resistant *S. stoloniferum* 17605-4 that contained the conserved *Rpi-blb1* homologue and the susceptible breeding clone RH89-039-16 were evaluated for late blight resistance. This test resulted in 19 resistant and 14 susceptible genotypes, indicating that a single dominant R gene segregated in the population, which was designated as *Rpi-sto1*. Four primer pairs for *Rpi-blb1* (BLB1F/R, 517/1519, 1521/518, 1+1') were tested on all the individuals of the population, and all amplified fragments of the expected size co-segregated with the resistance. Figure 2 illustrates co-segregation between the BLB1F/R primer and the resistance. Subsequent digestion of amplicons of marker CT88 with the restriction enzyme *Hinf*I, showed that CT88 co-segregated with *Rpi-sto1* in repulsion phase (Fig. 3), suggesting that *Rpi-sto1* is located on chromosome VIII at a similar position as *Rpi-blb1* in *S. bulbocastanum* (van der Vossen et al. 2003).

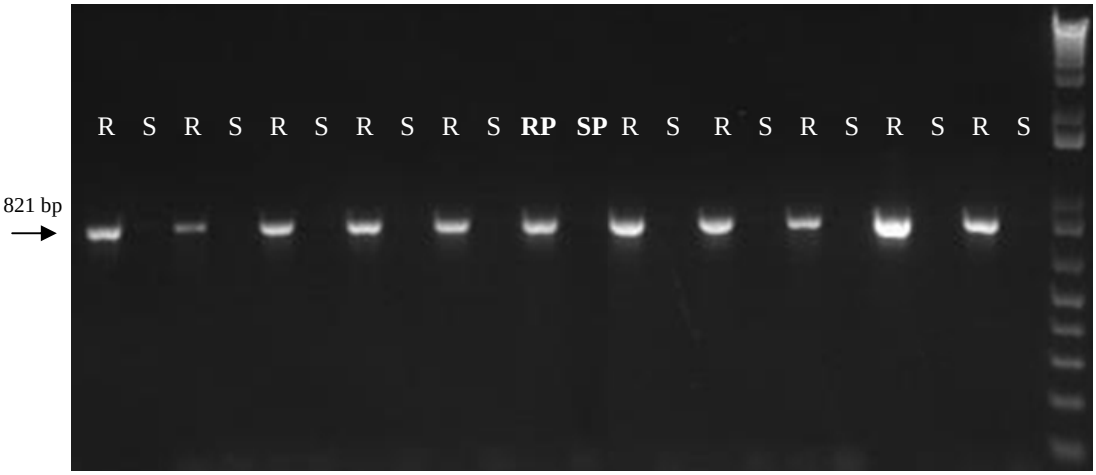


**Fig. 2** PCR amplification of primer BLB1F/R co-segregating with the resistance. Product size is 821 bp indicated by arrow. R, S, RP and SP indicate the resistant offspring, susceptible offspring, resistant parent *S. stoloniferum* CGN17605 genotype 4, susceptible parent RH89-039-16, respectively. A marker-size ladder is indicated (1kb+).

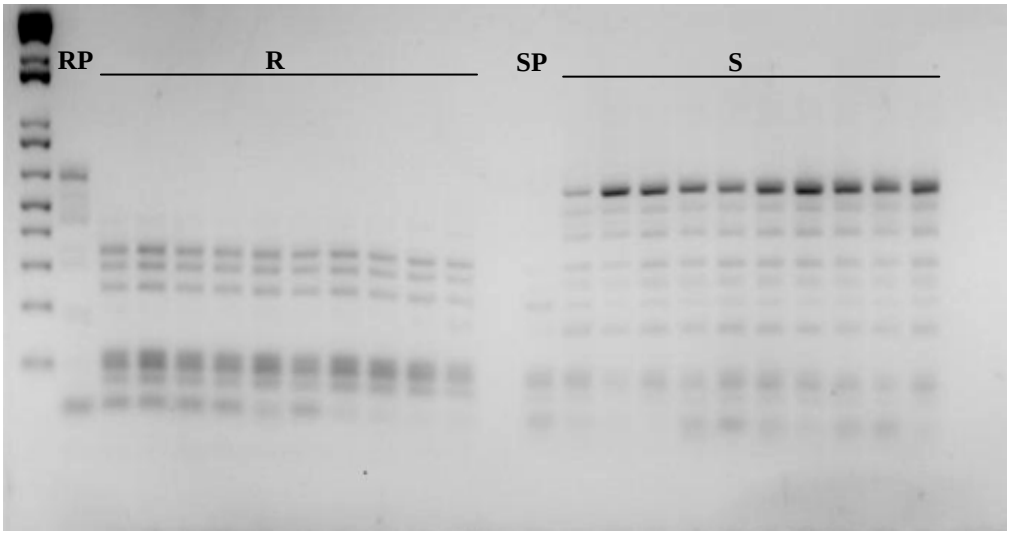


**Fig. 3** Linkage of *Rpi-sto1* to RFLP marker CT88. The polymorphism is revealed by restriction digestion with *Hinf I*. A marker-size ladder is indicated (1kb+). RP means resistant parent *S. stoloniferum* CGN17605 genotype 4, followed by ten resistant offspring. SP represents the susceptible parent RH 89-039-16, followed by ten susceptible offspring.

Forty offspring clones of each of the populations Pta 04-323, Pta 04-325 and Plt 04-281 were tested in triplicate in two independent DLAs with *P. infestans* isolates 655-2A and "Marknesse". All three populations segregated for resistance to late blight in these assays and for 36, 39 and 37 individuals respectively, a clear phenotype could be determined. The percentages of resistant offspring in the groups were 66, 69 and 73% respectively. For all three populations, the *Rpi-blb1* primer pairs BLB1F/R, 517/1519, 1521/518 and 1+1'(Table 2) produced positive results for some resistance genotypes, while other resistance genotypes produced negative results. None of the susceptible genotypes was positive for any of the *Rpi-blb1* related primers. The fact that all three populations consisted of about 75% resistant offspring, strongly suggests that there are two different R-genes segregating, one of which is highly homologous to *Rpi-blb1*, designated as *Rpi-plt1*, *Rpi-pta1* and *Rpi-pta2*, respectively, and the other an unknown late blight R gene.

*Hinf*I and *FspB*I digestion of CT88 PCR amplicons in population Plt 04-281 showed this marker to be genetically linked in coupling phase with resistance (Fig. 4), confirming that *Rpi-plt1* is also located on chromosome VIII.

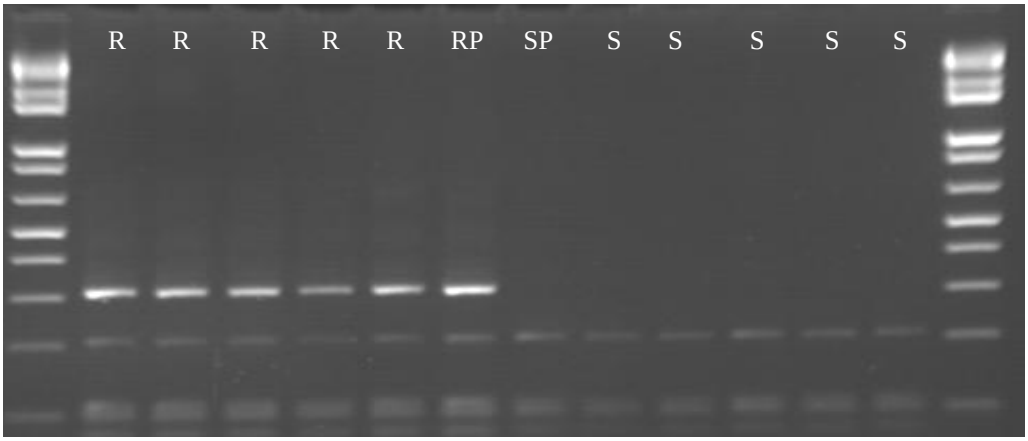


**Fig. 4** Linkage of *Rpi-plt1* to RFLP marker CT88. The polymorphism is revealed by restriction digestion with *Hinf I*. Marker-size ladders are indicated on the left and right border of the gel (1kb+). RP means resistant parent Plt 04-281, followed by five resistant offspring. SP represents the susceptible parent *S. polytrichon* CGN17751, followed by five susceptible offspring.

### ***Rpi-blb2*** **in** ***Solanum*** **species**

Using the primer pair BLB2F/R, *Rpi-blb2* homologues were not detected in any of the wild species clones evaluated (Table1, Table 3), but the positive control from which the gene was cloned was indeed found to contain *Rpi-blb2*. For some genotypes both degenerate primer pairs amplified fragments, while for some other genotypes only one degenerated primer pair amplified fragments. Sequence analysis from selected fragments showed that they had a low degree of homology to the sequence of *Rpi-blb2* (data not shown).

## Discussion

### Genomic organization of the *Rpi-blb1* gene cluster

Many R genes are present within clusters of tightly linked genes (Michelmore and Meyers 1998; Meyers et al. 2003). This is also the case for the *Rpi-blb1* gene (van der Vossen et al. 2003). Investigation of the presence or absence of the three paralogues *Rpi-blb1*, *RGA1-blb* and *RGA3-blb* showed that *RGA1-blb* homologues are present not only in all the tuber-bearing *Solanum* genotypes but also in non-tuber-bearing species (Table 1), suggesting that *RGA1-blb* was present before the divergence of tuber-bearing and non-tuber-bearing *Solanum* species. All 56 *RGA1-blb* fragments sequenced were highly homologous (96%-99% sequence identity with *RGA1-blb*), indicating that *RGA1-blb* is well conserved. As *RGA1-blb* is likely to be expressed (van der Vossen et al. 2003), its presence in such a wide variety of species

suggests that it might be a functional R gene and that it may confer resistance to a very common pathogen. Interestingly, whenever the conserved *Rpi-blb1* gene fragments were present in certain genotypes, both *RGA1-blb* and *RGA3-blb* homologues were also present (Table 1). On the contrary, genotypes that contained both *RGA1-blb* and *RGA3-blb* homologues did not necessarily contain the conserved *Rpi-blb1* gene homologue. *S. berthaultii* CGN 20644 and *S. chacoense* CGN18248 are two examples of accessions that contain homologues of both RGA1-blb and RGA3-blb, but not of *Rpi-blb1*, as judged from the amplification results with *Rpi-blb1* specific and degenerated primers. The presence of *RGA1-blb* and *RGA3-blb* homologues (or their ancestors) seems to be essential for the generation of the *Rpi-blb1* gene homologue, as was suggested previously (van der Vossen et al. 2003).

**Allele mining for homologues of *Rpi-blb1* and *Rpi-blb2***

Four species (*S. stoloniferum, S. papita, S. polytrichon, S. fendleri*) in the series *Longipedicellata* as recognized by Hawkes (1990) have been considered conspecific (Spooner et al. 2004), as both morphological characteristics (Spooner et al. 2001) and molecular data (van den Berg et al. 2002) failed to separate them. In this study conserved *Rpi-blb1* homologues were found in *S. bulbocastanum* and *S. stoloniferum* (sensu Spooner et al. 2004). In case of *S. stoloniferum* and *S. polytrichon* these *Rpi-blb1* homologues were confirmed to be linked to resistance and to the chromosome VIII specific marker CT88, which was previously shown to be linked to *Rpi-blb1* in *S. bulbocastanum*. All these data strongly suggest that *Rpi-sto1, Rpi-plt1, Rpi-pta1 and Rpi-pta2* are functional homologues of *Rpi-blb1*.

In contrast to *Rpi-blb1*, no conserved *Rpi-blb2* homologues were discovered in any of the genotypes evaluated, indicating that *Rpi-blb2* is probably a gene that has evolved relatively recently. However, it may be possible to find more *Rpi-blb1* and *Rpi-blb2* alleles as our screening was not exhaustive.

**Origin of *Rpi-sto1, Rpi-plt1, Rpi-pta1* and *Rpi-pta2***

The sequentially and positionally conserved *Rpi-blb1* homologues were found in the advanced polyploid Central American species *S. stoloniferum* (sensu Spooner et al. 2004) from series *Longipedicellata*, which is considered distinct from the primitive diploid species *S. bulbocastanum* from series *Bulbocastana* (Hawkes 1990; Spooner et al. 2004). Thus, the question arises how the genes *Rpi-sto1, Rpi-plt1, Rpi-pta1 and Rpi-pta2* ended up in *S. stoloniferum* (sensu Spooner et al. 2004). The Central American polyploid species from

series *Longipedicellata* are thought to have evolved from amphidiploidisations of a primitive Mexican ancestor with more advanced South American species (Hosaka et al. 1984; Hawkes 1990; Matsubayashi 1991). We propose two hypotheses. (1) *Rpi-blb1* is genetically highly conserved and was present in the wild ancestors of *S. stoloniferum* (sensu Spooner et al. 2004). *S. bulbocastanum* might be one of these ancestors. This hypothesis is supported by the similar constitution of the *Rpi-blb1* gene cluster (*RGA1-blb* and *RGA3-blb*) and the highly homologous sequences in *S. bulbocastanum* and *S. stoloniferum* (sensu Spooner et al. 2004) (Table 4). Furthermore, *Rpi-sto1* and *Rpi-plt1* were mapped in *S. stoloniferum* and *S. polytrichon* to the same chromosomal region of chromosome VIII as *Rpi-blb1* in *S. bulbocastanum*. (2) Alternatively, the *Rpi-blb1* homologues in *S. stoloniferum* (sensu Spooner et al. 2004) are the result of independent recombination events. However, the high level of sequence conservation of the *Rpi-blb1* homologues and previous cytogenetic studies (Hawkes 1990; Matsubayashi 1991) suggests the first alternative as being more likely.

Interestingly, resistance to root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax* is also found to be present in both *S. bulbocastanum* and *S. stoloniferum* (Janssen et al. 1996), indicating that other R genes in *S. stoloniferum* and *S. bulbocastanum* might share common ancestry too.

**Potato late blight breeding prospects**

The *Rpi-blb1* gene was originally discovered and cloned from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003 and 2005), a species that cannot be crossed with the cultivated potato *S. tuberosum* directly. Our study suggests that putatively functional *Rpi-blb1* homologues are also present in *S. stoloniferum* (sensu Spooner et al. 2004), a species that can be crossed with cultivated potato directly (Jackson and Hanneman. 1999), although the crossing efficiency is low. Assuming that the *Rpi-sto1* gene has the same specificity as *Rpi-blb1* it may now be easier to introduce the *Rpi-blb1* resistance specificity into cultivated potato from *S. stoloniferum* (sensu Spooner et al. 2004) instead of *S. bulbocastanum*. This is supported by the fact that *S. stoloniferum* (CPC 2093) has been used to breed potato varieties ‘Kuras’, ‘Santé’, ‘Xantia’ and ‘Lady Christl’ (Hutten and van Berloo 2001). Use of *S. bulbocastanum* is only possible through a tedious and time-consuming breeding scheme, for example, through bridge crosses (Hermsen and Ramanna 1973), or through somatic hybridization (Helgeson et al. 1998).

We anticipate that for other resistance genes present in primitive species, a similar situation may exist, i.e. homologues being present also in more advanced species that can be

more easily used for breeding. Therefore, before starting a breeding program with a species that does not allow an immediate cross with cultivated potato, evaluation of directly crossable germplasm for the presence of that gene may speed up the breeding program and save time and money.

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# Chapter 5

# Diversity and evolution of the late blight resistance genes *Rpi-blb1* and *Rpi-blb2* in *Solanum bulbocastanum* and *Solanum cardiophyllum*

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**Abstract**

Late blight is the most devastating potato disease in the world. Among the potato late blight resistance genes cloned so far, *Rpi-blb1* and *Rpi-blb2* originate from *Solanum bulbocastanum*. Both are claimed to confer resistance to a broad spectrum of *Phytophthora* isolates. We explored allele frequency and allelic diversity of *Rpi-blb1* and *Rpi-blb2* in 38 *S. bulbocastanum* and five *Solanum cardiophyllum* accessions. *Rpi-blb1* paralogues *RGA1-blb* and *RGA3-blb* were also examined. Conserved *Rpi-blb1* alleles were found in 24 Mexican accessions, but not in material originating from Guatemala. *Rpi-blb2* was present in eight *S. bulbocastanum* accessions only. Sequence analysis of a randomly selected set of genotypes revealed 19 different *Rpi-blb1* haplotypes. Our results confirm that *Rpi-blbl* belongs to the type II class of resistance genes that evolve slowly. Sequence analysis of the putative susceptible alleles of *Rpi-blb1* suggests that a single mutation event generated this susceptible allele. *Rpi-blb2* was identified at a low frequency only in *S. bulbocastanum* (12% of the genotypes), and all the identified alleles were identical. These data suggest that *Rpi-blb2* has evolved recently.

## Introduction

Late blight, caused by *Phytophthora infestans*, is the major disease in most potato growing areas. Mexico is the putative center of origin of *P. infestans*, but also of wild *Solanum* species, that are well known for their resistance to late blight, including *S. bulbocastanum*, *S. demissum* and *S. stoloniferum* (Toxopeus 1964; Howard, 1970; Umaerus, 1973; Ross, 1979; Hawkes 1990; Grunwald et al. 2005). Very recently, one study indicates an Andean origin of *Phytophthora infestans* as well, which was inferred from mitochondrial and nuclear gene genealogies (Gomez-Alpizar et al. 2007).

To date, three late blight resistance genes have been identified in *S. bulbocastanum*: the allelic genes *RB* and *Rpi-blb1* (Song et al. 2003; van der Vossen et al. 2003), *Rpi-blb2* (van der Vossen et al. 2005) and *Rpi-blb3* (Park et al. 2005). From these three genes, *Rpi-blb1* and *Rpi-blb2* have been cloned, and both are members of the NBS-LRR class of resistance genes. *Rpi-blb1* is present in a cluster of four resistance gene analogs on chromosome VIII and is flanked by *RGA1-blb* and *RGA3-blb* (Naess et al. 2000; van der Vossen et al. 2003). Two *Rpi-blb1* alleles, a "resistant" allele and a "susceptible" allele, were 99.8% similar with an 18-bp deletion present in the susceptible allele (Song et al. 2003). *Rpi-blb2* is in a locus harboring at least 15 *Mi-1* gene homologs on chromosome VI, and the *Rpi-blb2* protein shares 82% sequence identity to the *Mi-1* protein (van der Vossen et al. 2005). *Mi-1* is a gene of tomato that confers resistance to the root knot nematode *Meloidogyne incognita* (Milligan et al. 1998). The *Rpi-blb3* gene was identified on chromosome 4 in a gene cluster including three other genes *Rpi-abpt*, *R2*, and *R2*-like (Park et al. 2005).

The distribution of specific resistance genes in natural populations has been studied in a few cases only, for example, *RPS2* and *RPP13* in *Arabidopsis thaliana* (Mauricio et al. 2003; Rose et al. 2004), and *Cf-4* and *Cf-9* in tomato (Kruijt et al. 2005). To our knowledge, late blight resistance gene diversity studies have not been reported yet.

Song and colleagues (2003) suggest that *Rpi-blb1* shows an evolutionary pattern typical to a Type II resistance gene, which implies that *Rpi-blb1* is likely to be highly conserved in different genotypes or closely related species and present in high frequencies in natural populations (Song et al. 2003; Kuang et al. 2004). Distribution

of resistance genes in natural populations is not easy to study phenotypically, as resistance to one particular pathogen might result from the presence of one or more different resistance genes, which is the case for populations of *S. bulbocastanum*. One option is to use *Avr*-genes to determine whether a corresponding R-gene is present. However, at present, the corresponding *Avr*-genes for *Rpi-blb1* and *Rpi-blb2* are unknown, thus one needs to study the distribution of the genes using highly specific molecular markers and deduce the R-gene phenotype from these marker data.

In this paper, we studied the allelic frequencies of the *Rpi-blb1* and *Rpi-blb2* genes in accessions of two species, *S. bulbocastanum* and *S. cardiophyllum*, and the variation within them. Allelic frequencies of the two *Rpi-blb1* flanking genes *RGA1-blb* and *RGA3-blb* were also studied in some *S. bulbocastanum* genotypes. We related the occurrence of the genes *Rpi-blb1* and *Rpi-blb2* to the geographical origin of the accessions. Based on the presence/absence data of *Rpi-blb1* the evolutionary history of the gene is discussed.

## Materials and methods

### Plant material

Seventeen *S. bulbocastanum* accessions were obtained from the United States potato Genebank, in Sturgeon Bay, WI, USA, and kindly supplied as DNA samples by Dr. A. del Rio, Univ. Wisconsin, USA. Additionally, 21 *S. bulbocastanum* accessions and five *Solanum cardiophyllum* accessions, were obtained as seeds from the Centre for Genetic Resources, the Netherlands (CGN) (Table 1). Three to ten genotypes (Table 1) were analyzed from each accession. Individual seeds from CGN were surface-sterilized and sown in vitro on MS medium supplemented with 20% sucrose (Murashige and Skoog. 1962) at 18$^{o}$C and allowed to germinate for at least 6 weeks to obtain individual clones. DNA was extracted according to the method described by Stewart and Via (1993).

**Table 1** Distribution of *Rpi-blb1* and *Rpi-blb2* allele in accessions. Mexican accessions with coordinates were ordered according to the coordinates, followed by Mexican accessions without coordinates and those from Guatemala.

| | | | | | | | Rpi-blb1[c] | | | | Rpi-blb2[d] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Country | State | Taxon[a] | Genebank[b] | Coordinates | | Total | No.R | NO.S | Freq | Class | No | Freq |
| Mexico | Chiapas | ptt | G5323 | 16°10' N | 92°12' W | 3 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | Morelos | dph | G35399 | 19°01' N | 99°06' W | 4 | 0 | 2 | 0.00 | 1 | 0 | 0.00 |
| | Puebla | blb1 | P275190 | 19°04' N | 98°18' W | 3 | 0 | 3 | 0.00 | 1 | 0 | 0.00 |
| | Mexico | blb2 | C17691 | 19°13' N | 98°48' W | 4 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | Mexico | blb3 | C22367 | 19°13' N | 98°48' W | 5 | 0 | 5 | 0.00 | 1 | 0 | 0.00 |
| | Federal District | blb4 | P275185 | 19°21' N | 99°12' W | 7 | 0 | 4 | 0.00 | 1 | 0 | 0.00 |
| | Mexico | blb5 | B8009 | 19°29' N | 98°54' W | 4 | 0 | 1 | 0.00 | 1 | 0 | 0.00 |
| | Michoacan | blb6 | C17690 | 19°50' N | 101°43' W | 5 | 0 | 2 | 0.00 | 1 | 0 | 0.00 |
| | Federal District | blb7 | P275197 | 19°21' N | 99°12' W | 8 | 1 | 0 | 0.13 | 2 | 3 | 0.38 |
| | Veracruz | blb8 | P365379 | 18°43' N | 97°19' W | 8 | 2 | 0 | 0.25 | 2 | 0 | 0.00 |
| | Jalisco | blb9 | P590930 | 21°01' N | 102°59' W | 6 | 2 | 4 | 0.33 | 2 | 0 | 0.00 |
| | Distrito Federal | blb10 | C17689 | 19°21' N | 99°12' W | 4 | 2 | 2 | 0.50 | 2 | 0 | 0.00 |
| | Oaxaca | blb11 | P498223 | 17°02' N | 96°46' W | 9 | 5 | 0 | 0.56 | 2 | 9 | 1.00 |
| | Guerrero | blb12 | P545711 | 17°33' N | 99°30' W | 10 | 6 | 0 | 0.60 | 2 | 4 | 0.40 |
| | Michoacan | blb13 | P498225 | 19°25' N | 100°20' W | 8 | 5 | 3 | 0.63 | 2 | 0 | 0.00 |
| | Michoacan | blb14 | P347757 | 19°31' N | 100°15' W | 8 | 5 | 2 | 0.63 | 2 | 0 | 0.00 |
| | Oaxaca | blb15 | P283096 | 17°30' N | 96°46' W | 9 | 6 | 0 | 0.67 | 2 | 0 | 0.00 |
| | Jalisco | blb16 | P545751 | 20°59' N | 103°10' W | 7 | 5 | 0 | 0.71 | 2 | 0 | 0.00 |
| | Oaxaca | blb17 | P498011 | 17°02' N | 96°46' W | 9 | 7 | 1 | 0.78 | 2 | 8 | 0.89 |
| | Oaxaca | blb18 | C17692 | 17°53' N | 96°33' W | 5 | 4 | 1 | 0.80 | 2 | 2 | 0.40 |
| | Oaxaca | blb19 | P558377 | 16°16' N | 96°41' W | 7 | 6 | 0 | 0.86 | 2 | 0 | 0.00 |
| | Oaxaca | blb20 | P275195 | 17°02' N | 96°46' W | 6 | 6 | 0 | 1.00 | 3 | 2 | 0.33 |
| | Oaxaca | blb21 | C21306 | 17°30' N | 96°27' W | 4 | 4 | 0 | 1.00 | 3 | 0 | 0.00 |
| | Tlaxcala | blb22 | C22698 | 19°11' N | 98°13' W | 5 | 5 | 0 | 1.00 | 3 | 1 | 0.20 |
| | Mexico | blb23 | C17693 | 19°43' N | 99°47' W | 3 | 3 | 0 | 1.00 | 3 | 0 | 0.00 |
| | Michoacan | blb24 | P498224 | 21°13' N | 99°02' W | 10 | 10 | 0 | 1.00 | 3 | 0 | 0.00 |
| | nd[e] | cph | C18326 | nd | nd | 7 | 2 | 0 | 0.29 | 2 | 0 | 0.00 |
| | Distrito Federal | blb | C17687 | nd | nd | 5 | 2 | 2 | 0.40 | 2 | 0 | 0.00 |
| | Mexico | blb | C23010 | nd | nd | 5 | 3 | 1 | 0.60 | 2 | 0 | 0.00 |
| | Mexico | cph | C22387 | nd | nd | 5 | 3 | 0 | 0.60 | 2 | 0 | 0.00 |
| | Puebla | blb | P310960 | nd | nd | 7 | 7 | 0 | 1.00 | 3 | 0 | 0.00 |
| | nd | cph | B55227 | nd | nd | 3 | 3 | 0 | 1.00 | 3 | 0 | 0.00 |
| | nd | blb | C18310 | nd | nd | 5 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | nd | blb | B53682 | nd | nd | 5 | 0 | 3 | 0.00 | 1 | 0 | 0.00 |
| | Michoacan | cph | C18325 | nd | nd | 8 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | nd | cph | C17697 | nd | nd | 4 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| Guatemala | Baja Verapaz | blb | C21363 | 15°09' N | 90°18' W | 4 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | Huehuetenango | ptt | G5322 | 15°10' N | 91°31' W | 4 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | Baja Verapaz | blb | P604065 | 15°10' N | 90°17' W | 8 | 0 | 0 | 0.00 | 1 | 1 | 0.13 |
| | Baja Verapaz | blb | C21364 | 15°13' N | 90°18' W | 4 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | Huehuetenango | blb | C23075 | 15°19' N | 91°33' W | 4 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | Huehuetenango | blb | C22732 | 15°19' N | 91°31' W | 5 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | Huehuetenango | blb | C23074 | 15°46' N | 91°30' W | 5 | 0 | 0 | 0.00 | 1 | 0 | 0.00 |
| | | sum | | | | 249 | 104 | 36 | 0.42 | | 30 | 0.12 |

[a] Taxon abbreviations follow Hawkes (1990): blb = *S. bulbocastanum* subsp. *bulbocastanum*; dph = S. *bulbocastanum* subsp. *dolichophyllum*; ptt = *S. bulbocastanum* subsp. *partitum*; cph = *S. cardiophyllum* subsp. *cardiophyllum*. Taxon names for Mexican accessions with coordinates were used in Fig, 1.

[b] B represents Braunschweig Genetic Resources Collection (BGRC); C represents Center for Genetic Resources, the Netherlands (CGN); G represents Gross Lusewitz, Konrad Schuler (GLKS), Germany; P represents United States Department of Agriculture Plant Introduction

Numbers (PI).

[c] No. R: genotype numbers containing putative resistant (PR) *Rpi-blb1*; No. S: genotype numbers containing putative susceptible *Rpi-blb1* only; freq: frequency of the PR allele, freq=No.R/Total; Class: Accessions were grouped into three classes based on the freq (see results in the text for detail)

[d] No: genotype numbers containing *Rpi-blb2* determined by primer BLB2F/R; freq: frequency of *Rpi-blb2* containing materials in one accession

[e] nd: no data

**Amplification of resistance gene homologs and sequence analysis**

The primers used in this study were described by Wang et al. (Chapter 4). Three pairs of primers 517/1519, BLB1F/R, 1521/518 were used to amplify the *Rpi-blb1*. Primers 1 and 1' (Colton et al. 2006) were used to differentiate between the resistant and susceptible allele of *Rpi-blb1*. PCR conditions were as described by Colton et al. (2006). To determine the presence of the genes flanking *Rpi-blb1*, the primers RGA1F/R and RGA3F/R were used for *RGA1-blb* and *RGA3-blb*, respectively, on 23 randomly chosen genotypes from 20 accessions in *S. bulbocastanum*.

An additional primer set (BLB2F/R, Chapter 4) was used to amplify the *Rpi-blb2* gene. PCR reaction conditions and protocol were as described in Chapter 4. All amplification reactions were performed in a Biometra® T-Gradient or Biometra® Uno-II thermocycler (Westburg, Leusden, the Netherlands). PCR products were separated in 1.5% agarose gel and stained with ethidium bromide. The PCR reaction was repeated twice when no amplification product was observed.

For randomly selected genotypes, PCR products were sequenced directly to confirm their identity and to identify single nucleotide polymorphisms (SNPs). Each fragment was sequenced from both sides using the two primers as a sequencing primer with the BigDye Terminator kit. Sequencing reactions were analyzed using an ABI 3700 automated sequencer (Applied Biosystems, USA). DNA sequences were analyzed using DNAstar (Lasergene, Madison, WI, USA).

**Distribution of the examined materials**

Geographical origin (latitude and longitude information) of the accessions was collected from the website of CGN and from literature (Rodriguez and Spooner. 1997). Twenty-six Mexican accessions with detailed coordinates were located on the map

with the software DIVA-GIS (http://www.diva-gis.org).

## Results

### Detection of the *Rpi-blb1* gene

For the detection of the *Rpi-blb1* gene, three pairs of primers (517/1519, BLB1, 1521/518) were used, which showed congruent results: either all three produced a fragment or none did. When all three pairs of primers amplified fragments, the genotype was considered to contain the *Rpi-blb1* gene.

To investigate whether the resistant or susceptible allele was present, all genotypes were tested with the 1+1' primers (Colton et al. 2006). These results were confirmed by amplification and sequence analysis of fragments obtained with the primers 1521/518. Amplification with the 1+1' primer pair was only observed in the genotypes containing the *Rpi-blb1* gene. When both primer pairs (1521/518 and 1+1') amplified a fragment, the sequence derived from primer 1521/518 was either similar to that of the *Rpi-blb1* resistant allele or could only be read partly. In the latter case, the sequence reads were of high quality up to the point where the 18bp deletion starts, indicating heterozygosity of the locus. When there was no amplification for the 1+1' primers, the sequence derived from the primers 1521/518 was similar to the susceptible allele *rb*, i.e. containing the 18-bp deletion (Song et al. 2003). In these cases the material was considered to be homozygous for the putative susceptible (PS) allele. All genotypes showing positive results for primers 1+1' were considered to contain the putative resistant (PR) allele, either in heterozygous or homozygous state. Results of the analysis are summarized in Table 1.

### Distribution of the *Rpi-blb1* in accessions

Based on the presence or absence of the PR allele (Table 1), the forty-three populations were grouped into three classes. Class 1 contained 19 accessions, including 12 accessions without the *Rpi-blb1* gene in any of the genotypes examined and seven accessions where some or all the genotypes examined contained the PS allele. This class included all seven accessions from Guatemala. Class 2 contained 17 accessions, in which there was a mixture of putative resistant (containing the PR allele) and putative susceptible genotypes (either without the *Rpi-blb1* gene or containing the

PS allele). (3) Class 3 consisted of seven accessions, from which all genotypes examined contained the PR allele.

In total, 24 out of the 43 (approximately 56%) accessions contained the PR allele. In all the 249 genotypes examined the number of genotypes containing the PR allele, PS allele or a null allele was 104 (42%), 36 (14%) and 109 (44%), respectively.

**Sequence variation in the *Rpi-b*lb*1* gene**

To explore the R gene diversity at the nucleotide level, PCR amplicons from all three primer pairs (BLB1F/R, 517/1519 and 1521/518) were sequenced for 41 genotypes from 27 accessions. Four sequences were of bad quality due to the heterozygous state of the locus. These four sequences were excluded from further analysis. For the 37 genotypes from 26 accessions, DNA sequence variability was analyzed using the combined sequences from the three pairs of primers. In total, the aligned sequences shared by all PR genotypes spanned 1709 base pairs (bp), from the start codon to position 520 (the 517/1519 amplicon), and from position 2404 to the stop codon (overlapping BLB1F/R and 1521/519 amplicons) (Table 2), with the exception that an 18bp deletion was present in all the PS allele sequences. The length of the PR allele sequences was identical to that of the cloned *Rpi-blb1* gene, as were the PS allele sequences with that of the susceptible allele of *Rpi-blb1*. No insertion/deletion (indels) polymorphisms were found. All observed polymorphisms were point mutation.

Based on the DNA polymorphisms, the 28 PR sequences were classified into 19 haplotypes (Table 2). Within the 1709 bp, the maximum number of SNPs was eight. All *Rpi-blb1* homologous fragments showed 99.5 to 100% nucleotide identity with the cloned *Rpi-blb1* gene, suggesting that they are true alleles or orthologs of *Rpi-blb1*. Haplotype 19 was identical to *Rpi-blb1*. Alleles within the same accessions were identical or very similar. However, alleles from the same species were not always the most similar to each other. For example, alleles of haplotype 3 from *S. cardiophyllum* were more similar to the allele of haplotype 2 from *S. bulbocastanum* than to haplotype 16 from *S. cardiophyllum*. All nine PS sequences from six *S. bulbocastanum* accessions (two subspecies) were exactly the same, and they were also identical to the susceptible allele sequence described by Song et al. (2003). It is likely that the sample of alleles from *S. bulbocastanum* is not exhaustive, as many haplotypes were identified only once. The sampling of polymorphic positions was much more extensive, as only 9 out of 34 SNPs were found only once, 7 of which

**Table 2** Nucleotide polymorphisms in *Rpi-blb1* for putative resistant *Rpi-blb1* alleles

| Taxon[a] | Genebank | Genotype | Haplotype | 13 | 28 | 42 | 64 | 65 | 78 | 123 | 129 | 155 | 210 | 315 | 387 | 455[d] | 488 | 2407 | 2607 | 2745 | 2809 | 2818 | 2908 | 2981 | 3021 | 3134 | 3180 | 3255 | 3314 | 3376 | 3390 | 3442 | 3481 | 3517 | 3574 | 3588 | No. synonymous SNP[e] | No. non-synonymous SNP | No. of all SNP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Rpi-blb1*[b] | | | T | C | T | G | T | T | T | C | A | T | C | C | A | C | T | A | T | A | T | C | G | T | A | G | C | T | C | T | A | C | C | T | T | | | |
| blb | P590930 | 2 | 1 | G | T | | | C | | | | T | | | | | | C | G | | | | | | | | | | | | | | | | | C | | 3 | 4 | 7 |
| blb | C17693 | 10 | 2 | | Y | Y[c] | | | | Y | | | | | | | | | | G | | | | | | | | C | | | | | | | | | 4 | 1 | 5 |
| cph | B55227 | 1 | 3 | | T | G | | | | C | | | | | | | | | | G | | | | | | | | C | | | | | | | | | 4 | 1 | 5 |
| cph | B55227 | 4 | 3 | | T | G | | | | C | | | | | | | | | | G | | | | | | | | C | | | | | | | | | 4 | 1 | 5 |
| blb | P545751 | 1 | 4 | | T | | A | C | | C | | | C | | | | | | | | | | | | | | | | | | | | | | | A | 5 | 1 | 6 |
| blb | P498011 | 6 | 5 | | T | | | C | C | | | | | | | | | | | | | | | | | C | | | | | | | | | | A | 3 | 2 | 5 |
| blb | P498223 | 9 | 6 | | T | | | C | C | | | | | | | | | | | | | | | | | | | | | | | | | | | | 2 | 1 | 3 |
| blb | P347757 | 9 | 7 | | T | | | C | | | | | | | | | | | | G | | | | | C | C | | | T | | | | | | A | 2 | 5 | 7 |
| blb | P545711 | 1 | 8 | | T | | | C | | | | | | | | | | | | | | | | | | | | A | | | | G | | | | 2 | 2 | 4 |
| blb | P558377 | 1 | 9 | | T | | | C | | | | | | | | | | | | | | | | | | | | | | | | | T | T | | 3 | 1 | 4 |
| blb | P498224 | 1 | 10 | | T | | | C | | | | | | | | | | | | | | | | | | | | | | | | | | | | A | 1 | 2 | 3 |
| blb | P365379 | 10 | 11 | | T | | | C | | | | | | T | T | | | | | | | | | | | | | | | | | | | | | | 3 | 1 | 4 |
| blb | C23010 | 3 | 12 | | | | | C | | Y | | | | | | | | | | K | | Y | | | | | | | | | | | | | | | 2 | 2 | 4 |
| blb | P310960 | 2 | 13 | | | | | C | | | S | | | | | T | | | | | R | | | | S | | | S | | | | | | | | A | 3 | 3 | 6 |
| blb | P310960 | 5 | 14 | | | | | C | | | G | | | | | T | | | | | G | | | | C | | C | C | | | | | | | | A | 2 | 5 | 7 |
| blb | C17687 | 5 | 15 | | | | | C | | | | | | | | | | | | G | | C | T | | | C | | | T | C | | | | | | 3 | 4 | 7 |
| cph | C18326 | 10 | 16 | | | | | C | | | | | | T | | | | | | | | | | | | | | A | | | | | | | | | 1 | 2 | 3 |
| cph | C22387 | 1 | 16 | | | | | C | | | | | | T | | | | | | | | | | | | | | A | | | | | | | | | 1 | 2 | 3 |
| cph | C22387 | 4 | 16 | | | | | C | | | | | | T | | | | | | | | | | | | | | A | | | | | | | | | 1 | 2 | 3 |
| blb | P498223 | 3 | 17 | | | | | | | | | | | | | | G | | | | | | | | | C | | | | | | | | | A | 1 | 2 | 3 |
| blb | C21306 | 5 | 18 | | | | | | | | | | | | | | G | | | | | | | | | | | | | | | | | | | | 1 | 0 | 1 |
| blb | C17689 | 3 | 18 | | | | | | | | | | | | | | G | | | | | | | | | | | | | | | | | | | | 1 | 0 | 1 |
| blb | P275195 | 5 | 18 | | | | | | | | | | | | | | G | | | | | | | | | | | | | | | | | | | | 1 | 0 | 1 |
| blb | P283096 | 2 | 18 | | | | | | | | | | | | | | G | | | | | | | | | | | | | | | | | | | | 1 | 0 | 1 |
| blb | C21306 | 1 | 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 0 | 0 | 0 |
| blb | C21306 | 8 | 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 0 | 0 | 0 |
| blb | C17692 | 1 | 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 0 | 0 | 0 |
| blb | C17692 | 2 | 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 0 | 0 | 0 |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 54 | 44 | 98 |

[a] Nucleotides are numbered beginning at the start of the gene. The sequence spans 1709 base pairs (bp) in total, composed of two separate parts: (1) from the start codon to position 520 (taking *Rpi-blb1* as reference) derived from the primer 517/1519. In this part from the position 428 it is the intron sequence. (2) from position 2404 to stop codon derived from the primer BLB1F/R and 1521/519. Nucleotide changes are indicated by the appropriate letter. Grey background number suggests the SNP in that position changes the amino acid.

[b] *Rpi-blb1* sequence is used as reference for convenience only.

[c] Nucleotide abbreviations according to the IUB code (K, GT; R, AG; S , GC; Y, CT ) ); the three ‘haplotypes’ with these heterozygous positions may or may not be resolved into some of the other alleles upon cloning and sequencing.

[d] Underlined nucleotide means they are located in the intron.

[e] Number of SNP includes those from coding sequences only.

were found in two accession (P590930 and P545751) originating from the Jalisco in Mexico.

**Presence of *RGA1-blb* and *RGA3-blb***

Twenty-one genotypes, originating from 20 accessions, were characterized for the presence or absence of the two genes (RGA1-blb and RGA3-blb) flanking the Rpi-blb1 gene, covering accessions of three classes 1, 2 and 3. Interestingly, RGA1-blb and RGA3-blb were present in all 21 genotypes. Four RGA1-blb and four RGA3-blb derived fragments were sequenced, which were all highly similar within each gene (not shown). Alleles from RGA1-blb and RGA3-blb could be clearly distinguished from each other (data not shown).

**Distribution and sequence polymorphism of *Rpi-blb2***

In contrast to *Rpi-blb1*, BLB2F/R primers amplified fragments only from 32 genotypes. All fragments amplified from *S. bulbocastanum* had the expected size of 774bp, while a shorter fragment was amplified from two *S. cardiophyllum* genotypes originating from accession CGN22387.

To explore the variation in the *Rpi-blbl2* gene, PCR fragments from 16 genotypes (14 from *S. bulbocastanum* and two from *S. cardiophyllum*) were sequenced. All 14 sequences from *S. bulbocastanum* were identical to that of the cloned *Rpi-blb2*. The shorter sequences from *S. cardiophyllum* were possibly pseudogenes of *Rpi-blb2* due to the introduction of stop codons in the 5' end of the fragment (not shown). Therefore, only when primer BLB2F/R amplified the expected fragment size, did we consider the genotype to contain *Rpi-blb2*. In total, eight out of 43 accessions (about 19%), 30 out of 249 genotypes (12%) contained the *Rpi-blb2* (Table 1).

**Geographical distribution of *Rpi-blb1* and *Rpi-blb2***

Twenty-six Mexican accessions could be located on the map (Fig.1), while six accessions with some genotypes containing the PR allele could not be located due to missing coordinate information (Table 1). Geographically, populations containing the *Rpi-blb1* PR alleles were distributed more widely than those containing the *Rpi-blb2*. Interestingly, accessions without the *Rpi-blb1* gene also did not contain the *Rpi-blb2* gene, except for one *S. bulbocastanum* accession P604065 originating from

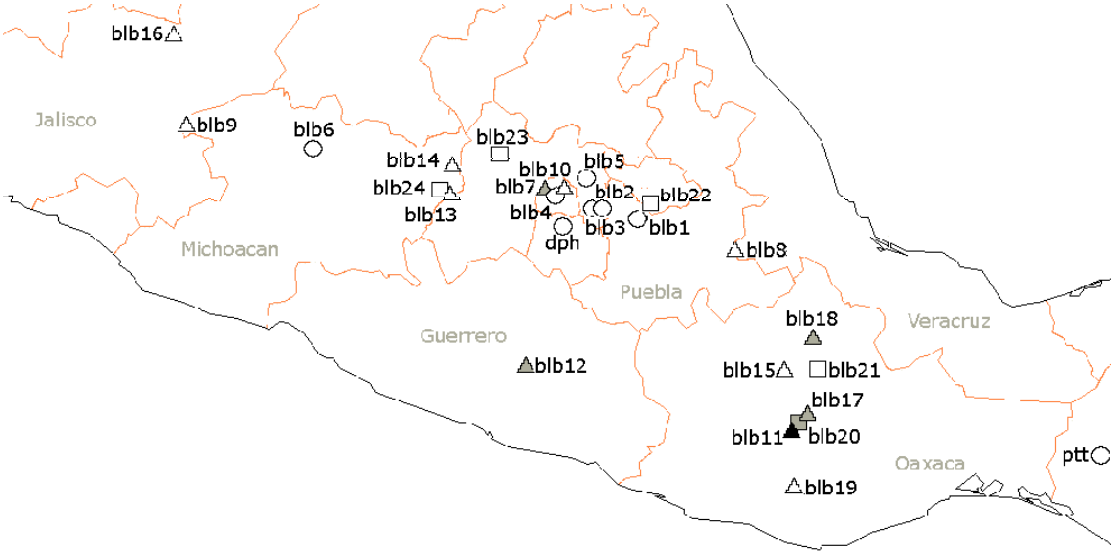


**Fig. 1** Population distribution in Mexico and gene frequency of the putative resistance gene *Rpi-blb1* and *Rpi-blb2* . Locations for populations having the same coordinates are edited manually to increase the readability in the map. Coordinates and other details are listed in Table 1. Figures in the map represent accessions in which:

○ neither putative resistance *Rpi-blb1* nor *Rpi-blb2* gene is present

△ some genotypes contain the putative resistance *Rpi-blb1* but not *Rpi-blb2*

▲ some genotypes contain both the putative resistance *Rpi-blb1* and *Rpi-blb2*

▲ some genotypes contain the putative resistance *Rpi-blb1* and all genotypes contain the *Rpi-blb2*

□ all the genotypes contain the putative resistance *Rpi-blb1* but none of them contain the *Rpi-blb2*

■ all the genotypes contain the putative resistance *Rpi-blb1* and some genotypes contain the *Rpi-blb2*

Guatemala. All material originating from the Oaxaca state showed a *Rpi-blb1* PR allele frequency higher than 0.5. The accessions containing the *Rpi-blb2* gene also originated mainly from the Oaxaca state.

## Discussion

### Distribution of the *Rpi-blb1* and *Rpi-blb2* resistance gene

Using gene specific primers for the *Rpi-blb1* and *Rpi-blb2* genes (Chapter 4) we were able to analyze the distribution of these genes in accessions of *S. bulbocastanum* and

*S. cardiophyllum*. The *Rpi-blb1* PR allele was found to be present in 67% of the Mexican accessions examined but not in any of the seven accessions from Guatemala. However, no clear center of origin was identified in Mexico, either. Accessions with and without *Rpi-blb1* are scattered all over (Table 1 and Fig 1), with some concentration in the Oaxaca state. In our previous study (Chapter 4) we reported on the presence of PR *Rpi-blb1* alleles in *S. stoloniferum* accessions CGN17605 and CGN17607, both are located in the state Michoacan (coordinates: 19°42' N, 101°07' W) in Mexico, and accession BGRC60465, also located in Mexico (19°07' N, 98°46' W). Taken together these data suggest that Mexico is the center of origin of the *Rpi-blb1* gene.

Some *S. bulbocastanum* accessions/genotypes contain both *Rpi-blb1* and *Rpi-blb2*. However, there was only one population (PI498223) in which all individuals contained the *Rpi-blb2* gene. Most of the populations containing *Rpi-blb2* also contained *Rpi-blb1*. The frequency of the *Rpi-blb1* was much higher than that of the *Rpi-blb2* gene, 42% versus 12% respectively. The number of alleles and the nucleotide diversity of *Rpi-blb*1 is also much higher than that of *Rpi-blb*2. These differences might be related to 'age' of the genes. It will be interesting to also include the presence/absence of the *Rpi-blb3* gene (Park et al. 2005) in this analysis once the gene is cloned. The results confirm that *S. bulbocastanum* is a rich source of late blight resistance gene (Budin 2002).

**Co-existence of putative resistant and susceptible alleles of *Rpi-blb1***

Co-existence of both PR and PS alleles of *Rpi-blb1* was observed in *S. bulbocastanum* accessions (Table 1). Interestingly, PS phenotypes for *Rpi-blb1*, as determined from the gene specific marker data, did not only originate from the presence of the PS allele of *Rpi-blb*1, but also to the presence of a null allele. Among the 249 genotypes analyzed, 36 genotypes contained the PS allele, but 109 genotypes contained the null allele, indicating that latter situation is more frequent. We interpret the lack of amplification as evidence for complete absence of the gene, rather than the occurrence of an SNP at one of the primer binding sites, as none of the three primer pairs amplified in these genotypes, while all three amplified when PS or PR alleles were detected. The absence of the entire *Rpi-blb*1 gene may well be the most primitive situation, as the absence is much more common than the presence of the PS allele, and as the PS allele did not contain any genetic variation, while PR alleles existed in many

allelic variants.

The coexistence of resistant and susceptible alleles might have ecological meaning, possibly in preventing the pathogen breaking the resistance too quickly. Long-lived co-existence of resistant and susceptible alleles was also found across the range of *Arabidopsis thaliana* ecotypes and both alleles frequently occurred together within natural populations (Stahl et al. 1999).

**Variation in the *Rpi-blb1* and *Rpi-blb2* resistance genes**

Sequence analysis showed 19 haplotypes of the PR alleles of *Rpi-blb1*. In contrast, all the PS allele sequences were identical. For the PS alleles, the SNP at the 454th codon causing the premature stop codon identified by Song et al. (2003) was not studied, as our primers do not cover that sequence. SNPs identified in the sequences (Table 2) are 22 synonymous SNPs, which do not alter the amino acids incorporated. The 12 non-synonymous SNPs involve the following amino acid changes but some of them possibly do not influence the function of the gene. This is already shown by our data in Chapter 4, where non-synonymous SNPs in position 64, 65 (both code for the same amino acid, from Val to Thr), 3134 (from Met to Leu) and 3588 (from Ile to Asn) are present, but the plants show resistance to late blight. Therefore, we anticipated that the 19 haplotypes identified are putatively functional genes.

As far as there is overlap, all the PS allele sequences were identical with the susceptible allele reported by Song et al. (2003), suggesting that all PS allele sequences originate from a single mutation event that might have generated this susceptible allele relatively recently. Previously, we investigated the allele frequency of *Rpi-blb2* in more than 40 *Solanum* species by using the same BLB2F/R primer pair, but none of them contained *Rpi-blb2* (Chapter 4). Also in this study the frequency of the *Rpi-blb2* in *S. bulbocastanum* was low (12%) and the sequence of all fragments obtained was identical. The finding suggests that *Rpi-blb2* has evolved recently.

***Rpi-blb1* is a Type II resistance gene**

The rate of evolution of NBS-LRR-encoding genes can be rapid or slow. In lettuce, two types of RGC2 genes (Type I and Type II) were distinguished based on the pattern of sequence identity between sequence exchanges and their prevalence in natural populations (Kuang et al. 2004). Sequence exchanges are frequent between Type I genes, therefore, obscured allelic/orthologous relationships are observed. In

addition, due to the frequent sequence exchanges, individual Type I genes comprise diverse chimeras and are rare in natural populations. In contrast, Type II RGC2 genes are highly conserved, evolving slowly and maintaining obvious allelic/orthologous relationships between clades (Kuang et al. 2004). Point mutations are found to be the most common polymorphism between Type II alleles/orthologs.

All PR alleles of *Rpi-blb1* amplified from *S. bulbocastanum* and *S. cardiophyllum* (Table 2) and others from *S. stoloniferum* (Chapter 4) were 99.5 to 100% homologous to each other and to the *Rpi-blb1* gene, showing that PR alleles are clearly orthologs. In addition, where studied, genes flanking the original *Rpi-blb1* gene (*RGA1-blb* and *RGA3-blb*) were also present and highly homologous in all genotypes containing *Rpi-blb1* (Chapter 4). *S. bulbocastanum* and *S. cardiophyllum* are primitive diploid species, distinct from the polyploid *S. stoloniferum* (Hawkes 1990). Taken together our studies indicate that *Rpi-blb1* is present in both systematically close and distinct materials, that all *Rpi-blb1* alleles are highly homologous (>99.5% sequence identity), and that mainly point mutations contribute to the variation. All this confirms the Type 2 nature of *Rpi-blb1*.

Finding from this study together with those from our previous one (Chapter 4) enable us to conclude that *Rpi-blb1* belongs to Type II resistance gene. Taken with the finding that *Rpi-blb2* has evolved recently, our data contributed the understanding for the evolution of the two late blight resistance genes.

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# Chapter 6

# General discussion

## NBS profiling for systematics and identification of candidate R gene markers in wild and cultivated *Solanum* species

Potato (*Solanum tuberosum* L.) is a crop with a large secondary gene pool, which contains many important traits that can be exploited in breeding programs. As the number of species is large, it is not easy to choose the right material for variety improvement. Also, not all wild species are easily crossable with the cultivated potato (Hawkes 1990).

Insight in the systematic relationships within the tuber-bearing *Solanum* species might help to identify the most interesting materials for breeding purposes. Relationships within this group of species have been studied extensively, using morphological characteristics and also different molecular markers (Hosaka et al. 1984; Kardolus et al. 1998; Bryan et al. 1999; Miller et al. 1999; Volkov et al. 2001; Raker and Spooner 2002; Volkov et al. 2003; Sukhotu et al. 2004). However, the phylogenies obtained with neutral markers like AFLP, RFLP and chloroplast DNA have not resulted in a priori identification of the most promising species for resistance. Apparently there is not a good correlation between these neutral marker-based relationships and the occurrence of disease resistance genes. Functional markers based on diversity in resistance genes might be more predictive. Nucleotide binding site (NBS) profiling is a new marker technique that targets resistance genes and their analogs. The technique is based on selective amplification, using primers designed upon conserved motifs of cloned R genes. The fragments (markers) obtained are highly enriched for resistance gene analogs. The results obtained from NBS profiling data were compared to those from AFLP. Results from cladistic and phenetic analyses showed that the two techniques delivered trees with a similar topology and resolution, indicating that NBS profiling can be an alternative for phylogeny reconstruction. However, no clear effects of targeting resistance genes were visible in the NBS profiling tree. This congruence between AFLP and NBS profiling may not be as unexpected as it appears to be. Plants have to deal with many different pathogens during their lifetime and are thus exposed to selection pressures in different directions. However, it is possible that a single resistance gene is essential for species survival or a speciation event. The R-gene(s) that may have been under selection after a period of

disease pressure by a pathogen may have spread relatively fast to neighboring species through hybridization. The specific effect of selective pressure on R-genes will therefore be only detectable on a very short evolutionary time scale, and is diluted when many markers are analyzed phylogenetically. The outcome of the phylogenetic analysis is based on all NBS profiling markers, the majority of which was not affected by selective pressure. A single or a few markers very likely will not influence the outcome. All this makes that NBS profiling, although at least as effective as AFLP for phylogeny reconstruction, does not have a specific advantage in highlighting R-gene related phylogenies. It does have the advantage of low levels of homoplasy (Chapter 3). Co-migrating NBS profile marker bands were sequenced to check the identity of the bands. Within the tuber-bearing *Solanum* species, 91% of the sequences of co-migrating bands were more than 95% homologues to each other, indicating low levels of homoplasy.

Chapter 2 also shows that NBS profiling can be used to identify candidate resistance markers. By sequencing of species-specific markers, one allelic variant of the *Rpi-blb*2 gene was discovered in *Solanum bulbocastanum*. The use of NBS profiling for identifying markers linked to resistance genes was further explored in Chapter 3. Several markers, potentially linked to introgressed R-genes, are identified by linking NBS profiling bands with pedigree data and phenotypic data of the varieties. One of the resistance markers identified was very likely introgressed from *Solanum vernei*. This was indicated by the correlation between the presence of this marker and the resistance data for the varieties involved, as well as the presence of the same marker in both *S. vernei* accessions examined. This marker is present not only in the two *S. vernei* accessions, but also in two *S. microdontum* accessions. Systematically, both *S. vernei* and *S. microdontum* belong to a group of species in series *Tuberosa*, occurring in Bolivia and Argentina. The presence of this marker in both species may indicate a common ancestry of this marker.

An alternative approach for finding resistance markers in a set of varieties is association mapping, as reported by Malosetti et al. (2007). Using a subset of the varieties used in Chapter 3 and the same NBS profiling marker data, two markers were identified with significant association to late blight resistance using a mixed model approach. Previously, marker-trait associations were successfully used to assess the genetic potential of the potato germplam collections (Gebhardt et al. 2004). In their study, in which a collection of 600 varieties and various wild species was used,

DNA markers linked to previously mapped QTLs for resistance to late blight and plant maturity were evaluated. Highly significant association with QTL for resistance to late blight and plant maturity was detected with PCR markers specific for R1. In addition, this marker was traced to an introgression from the wild species *S. demissum*. Molecular and late blight phenotype data showed that the R1 marker was also present in another wild species *S. stoloniferum* (Gebhardt et al. 2004), indicating that R1 containing materials cannot with a 100% certainty be traced back to *S. demissum*.

In Chapter 3 we also assessed the changes in genetic diversity during the last 70 - 80 years in a set of about 460 European potato varieties using NBS profiling. Analysis showed that during that period diversity in markers linked to resistance had increased. Only one marker was lost, whereas several new markers were introduced into the potato gene pool, which most likely reflects the efforts of breeding companies and research institutes to introgress resistances from wild species into cultivated potato.

**Late blight R genes in cultivated and wild *Solanum* species**

Recently, four late blight R genes have been cloned from potato. All these encode a coiled coil (CC)-NBS-LRR class of proteins, including R1 (Ballvora et al. 2002) and R3a (Huang et al. 2005) from *S. demissum* and *RB/Rpi-blb1* and *Rpi-blb2* (Song et al. 2003; van der Vossen et al 2003; van der Vossen et al 2005) from *S. bulbocastanum*.

Allelic frequency and variation of *Rpi-blb1* and *Rpi-blb2* was analyzed in a large number of tuber-bearing *Solanum* species. The genomic organization of the *Rpi-blb1* cluster in these species was also explored. *Rpi-blb*1 and *Rpi-blb*2 were chosen as they confer resistance to all the *P. infestans* isolates tested so far. Insight into allelic diversity may facilitate discovery of functional homologues that can be exploited in breeding programs and may also help to understand the evolution of R-genes.

Highly specific primers were designed to selectively amplify the R-gene under study. In this way, we expect to amplify R gene orthologs only and exclude the paralogs. PCR amplifications from several pairs and sequence analysis provide an overview of the presence of the two R genes. *Rpi-blb*1 candidates were discovered in two species besides *S. bulbocastanum: S. stoloniferum* (sensu Spooner et al. 2001; Chapter 4) and *S. cardiophyllum* (Chapter 5). The genes *Rpi-sto1, Rpi-pta1, Rpi-pta2,* and *Rpi-plt1* are putative functional homologs of *Rpi-blb*1. An analysis using four segregating populations confirmed that the genes all mapped on potato chromosome

VIII. *Rpi-blb*1 was also mapped on this chromosome in *S. bulbocastanum*. In addition to the identification of *Rpi-blb*1, three out of four segregating populations examined also contained another unknown late blight resistance gene (Chapter 4). Although the detailed characterization of these genes is not available yet and it is unknown whether these genes are similar to other known R-genes or that they are new ones, there is no doubt that these resistant clones represent promising material for breeding. As a first step these genes should be mapped.

In contrast to the presence of *Rpi-blb*1 in *S. bulbocastanum, S. cardiophyllum* and *S. stoloniferum, Rpi-blb*2 was found only in accessions from species *S. bulbocastanum*. Partial sequences derived from the amplified fragments are all identical to the *Rpi-blb*2 gene. Our observation that the presence of *Rpi-blb*2 is restricted to *S. bulbocastanum* and that the sequences of all fragments obtained are identical suggests that *Rpi-blb*2 evolved recently.

**Presence of *Rpi-blb*1 and other R genes in more than one species**

As described in Chapter 4 and 5, *Rpi-blb*1 is present in *S. bulbocastanum, S. cardiophyllum* and *S. stoloniferum*. By chance or not, the resistance to root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax* was also found to be present in *S. bulbocastanum, S. cardiophyllum* and *S. stoloniferum* (Janssen et al. 1995). Genes R2, R3, R4 and R6 are also identified in *S. stoloniferum* (McKee 1962). In addition, R6 was recorded in *S. stoloniferum* before it was recognized in *S. demissum* (Black 1960). Recently, the *R1* is reported to be present in *S. demissum* as well as in *S. stoloniferum* (Gebhardt et al. 2004). *S. bulbocastanum* and *S. cardiophyllum* are considered as primitive diploid species in the series *Bulbocastana* and *Pinnatisecta*, respectively, distinct from the advanced tetraploid Central American species *S. stoloniferum* in the series *Longipedicellata* (Hawkes 1990). The Central American polyploid species from series *Demissa* and *Longipedicellata* are thought to have evolved from amphidiploidizations of primitive Mexican ancestors with more advanced South American species (Hosaka et al. 1984; Hawkes 1990; Matsubayashi 1991). The low number of polymorphisms among *Rpi-blb1* homologs in *S. bulbocastanum, S. cardiophyllum* and *S. stoloniferum* indicates that all these genes from different species may share one common ancestry, and that this gene was already present in the ancestors of the current *Solanum* gene pool. It also suggests that *S. bulbocastanum* might be one of the parents of the Mexican polyploid species

*S. stoloniferum*. This is also supported by the fact that not only *Rpi-blb1* but also the gene cluster members *RGA1-blb* and *RGA3-blb* are conserved in the species (Chapter 4).

**Perspectives for resistance breeding**

*Rpi-blb*1 confers a resistance against various known *P. infestans* strains (Song et al. 2003; van der Vossen et al. 2003). This suggests that *Rpi-blb*1may have a different molecular mechanism against the late blight pathogen when compared to the other R genes from *S. demissum*. Therefore, *Rpi-blb*1 may provide more durable and effective late blight resistance for potato breeding (Colton et al. 2006). Other homologous R genes (*Rpi-sto1, Rpi-pta1, Rpi-pta2 and Rpi-plt1*) of *Rpi-blb*1 in *S. stoloniferum* (Chapter 4) may also confer broad-spectrum resistance to late blight.

The *Rpi-blb1* gene was originally discovered and cloned from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003 and 2005), a species that cannot be crossed with the cultivated potato *S. tuberosum* directly. Our study showed that a functional homolog of *Rpi-blb1* is also present in *S. stoloniferum*, which can be crossed with cultivated potato directly (Jackson and Hanneman 1999), although the crossing efficiency is low. This is supported by the fact that *S. stoloniferum* (CPC 2093) has been used to breed potato varieties (Hutten and van Berloo 2001). So, the *Rpi-sto1* gene from *S. stoloniferum* should be easier to introduce into cultivated potato than the *Rpi-blb1* gene from *S. bulbocastanum*. In addition, making use of *S. bulbocastanum* is only possible through a tedious and time-consuming breeding scheme, for example, through bridge crosses (Hermsen and Ramanna 1973) or through somatic hybridization (Helgeson et al. 1998).

As mentioned above, several R genes against late blight and root-knot nematode are present in *S. stoloniferum*. Therefore, extensive screening of *S. stoloniferum* may identify material which confers resistance to other diseases as well. This material can be used for multi-traits resistance breeding in potato.

# References


Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST, a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402

Andersson B, Sandstrom M, Stromberg A (1998) Indications of soil borne inoculum of *Phytophthora infestans.* Pot Res 41:305-310

Bakker E, Butterbach P, Rouppe van der Voort J, van der Vossen E, van Vliet J, Bakker J, Goverse A (2003) Genetic and physical mapping of homologues of the virus resistance gene Rx1 and the cyst nematode resistance gene Gpa2 in potato. Theor Appl Genet 106: 1524-1531

Ballvora, A, Ercolano MR, Weiss J, Meksem K, Bormann CA, Oberhagemann P, Salamini F, Gebhardt C (2002) The R1 gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. Plant J 30: 361-371

Barker FK, Lutzoni FM (2002) The utility of the incongruence length difference test. Syst Biol 51: 625-637

Barone A, Ritter E, Schachtschabel U, Debener T, Salamini F, Gebhardt C (1990) Localization by restriction fragment length polymorphism mapping in potato of a major dominant gene conferring resistance to the potato cyst nematode *Globodera rostochiensis.* Mol Gen Genet 224: 177-182

Bendahmane A, Kanyuka K, Baulcombe DC (1997) High-resolution genetical and physical mapping of the Rx gene for extreme resistance to potato virus X in tetraploid potato. Theor Appl Genet 95:153-162

Birch PRJ, Whisson SC (2001) *Phytophthora infestans* enters the genomics era. Mol Plant Pathol 2: 257-263

Bisognin DA, Douches DS, Buszka L, Bryan G, Wang D (2005) Mapping late blight resistance in *Solanum microdontum* Bitter. Crop Sci 45: 340-345

Black W (1960) Races of *Phytophthora infestans* and resistance problems in potatoes. Scottish plant breeding station rept: 29-38

Black W, Mastenbroek C, Mills WR, Peterson LC (1953) A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives. Euphytica 2: 173-179

Bourke A (1993) The visitation of god? The potato and the great Irish famine. Lilliput Press Ltd, Dublin

Bradshaw JE, Bryan GJ, Lees AK, McLean K, Solomon-Blackburn RM (2005) Mapping the R10 and R11 genes for resistance to late blight *(Phytophthora infestans*) present in the potato *(Solanum tuberosum*) R-gene differentials of Black. Theor Appl Genet 112: 744-751

Bradshaw JE, Bryan GJ, Ramsay G (2006) Genetic Resources (Including wild and cultivated *Solanum* Species) and progress in their utilisation in potato breeding. Pot Res 49: 49-65

Bryan GJ, Mcnicoll J, Ramsay G, Meyer RC, De Jong WS (1999) Polymorphic simple sequence repeat markers in chloroplast genomes of Solanaceous plants. Theor Appl Genet 99: 859-867

Budin KZ (2002) Genetic foci of *Solanum* species, *Petota* Dumort, resistant to *Phytophthora infestans* (Mont.) De Bary. Genetic resources and crop evolution 49: 229-235

Calenge F, van der Linden CG, van de Weg E, Schouten HJ, van Arkel G, Denance C, Durel CE (2005) Resistance gene analogues identified through the NBS-profiling method map close to major genes and QTL for disease resistance in apple. Theor Appl Genet 110: 660-668

Caromel B, Mugniéry D, Lefebvre V, Andrzejewski S, Ellissèche D, Kerlan MC, Rousselle P, Rousselle-Bourgeois F (2003) Mapping QTLs for resistance against *Globodera pallida* (Stone) Pa2/3 in a diploid potato progeny originating from *Solanum spegazzinii*. Theor Appl Genet 106: 1517-1523

Celebi-Toprak F, Slack SA, Jahn MM (2002) A new gene, $Ny_{tbr}$, for hypersensitivity to potato virus Y from *Solanum tuberosum* maps to chromosome IV. Theor Appl Genet 104: 669-674

Colton LM, Groza HI, Wielgus SM, Jiang J (2006) Marker-assisted selection for the broad-spectrum potato late blight resistance conferred by gene RB derived from a wild potato species. Crop Sci 46: 589-594

Darlu P, Lecointre G (2002) When does the incongruence length difference test fail? Mol Biol Evol 19: 432-437

Debener T, Salamini F, Gebhardt C (1990) Phylogeny of wild and cultivated *Solanum* species based on nuclear restriction fragment length polymorphisms (RFLPs). Theor Appl Genet 79: 360-368

DIVA-GIS (http://www.diva-gis.org)

Drenth A, Janssen EM, Govers F (1995) Formation and survival of oospores of *Phytophthora infestans* under natural conditions. Plant Pathol 44: 86-94

El-Kharbotly A, Palomino-Sánchez C, Salamini F, Jacobsen E, Gebhardt C (1996) R6 and R7 alleles of potato conferring race-specific resistance to *Phytophthora infestans* (Mont.) de Bary identified genetic loci clustering with the R3 locus on chromosome XI. Theor Appl Genet 92: 880-884

El-Rabey HA, Badr A, Schafer-Pregl R, Martin W, Salamini F (2002) Speciation and species separation in *Hordeum* L. (Poacease) resolved by discontinuous molecular markers. Plant Boil 4: 567-575

Ewing EE, Simko I, Smart CD, Bonierbale MW, Mizubuti ESG, May GD, Fry WE (2000) Genetic mapping from field tests of qualitative and quantitative resistance to *Phytophthora infestans* in a population derived from *Solanum tuberosum* and *Solanum berthaultii*. Mol Breed 6: 25-36

Farris JS, Källersjö M, Kluge AG, Bult C (1995) Testing significance of incongruence. Cladistics 10: 315-319

Flier WG, van den Bosch GBM, Turkensteen LJ (2003) Stability of partial resistance in potato cultivars exposed to aggressive strains of *Phytophthora infestans*. Plant Path 52: 326-337

Fry WE, Goodwin SB, Matuszak JM, Spielman LJ, Milgroom MG (1992) Population genetics and intercontinental migrations of *Phytophthora infestans*. Annu Rev Phytopathol 30: 107-129

Gaston KJ, Williams PH (1996) Spatial patterns in taxonomic diversity. In: Gaston KJ [eds], biodiversity, a biology of numbers and difference, Blackwell Science, London, pp 202-229

Gebhardt C, Ballvora A, Walkemeier B, Oberhagemann P, Schüler K (2004) Assessing genetic potential in germplasm collections of crop plants by marker-trait association: a case study for potatoes with quantitative variation of resistance to late blight and maturity type. Mol Breed 13: 93-102

Gebhardt C, Mugniéry D, Ritter E, Salamini F, Bonnel E (1993) Identification of RFLP markers closely linked to the H1 gene conferring resistance to *Globodera rostochiensis* in potato. Theor Appl Genet 85: 541-544

Gebhardt C, Valkonen JP (2001) Organization of genes controlling disease resistance in the potato genome. Annu Rev Phytopathol 39: 79-102

Ghislain M, Trognitz B, Herrera MR, Solis J, Casallo G, Vásquez C, Hurtado O, Castillo R, Portal L, Orrillo M (2001) Genetic loci associated with field resistance to late blight in offspring of *Solanum phureja* and *S. tuberosum* grown under short-day conditions. Theor Appl Genet 103:433-442

GILB (2004) Global initiative on late blight, http://www.cipotato.org/gilb/

Gomez-Alpizar L, Carbone I, Ristaino JB (2007) An Andean origin of *Phytophthora infestans* inferred from mitochondrial and nuclear gene genealogies. Proc Natl Acad Sci USA 104:3306-3311

Goodwin SB, Spielman LJ, Matuszak JM, Bergeron SN, Fry WE (1992) Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. Phytopathology 82: 955-961

Graham SW, Kohn JR, Morton BR, Eckenwalder JE, Barrett SCH (1998) Phylogenetic congruence and discordance among one morphological and three molecular data sets from Pontederiaceae. Syst Biol 47: 545-567

Grunwald NJ, Flier WG (2005) The biology of *Phytophthora infestans at* its center of origi*n*. Annu Rev Phytopathol 43: 171-190

Grunwald NJ, Flier WG, Sturbaum AK, Garay-Serrano E, van den Bosch TBM (2001) Population structure of *Phytophthora infestans* in the Toluca valley region of Central Mexico. Phytopathology 91: 882-890

Grunwald NJ, Romero MG, Lozoya SH, Rubio-Covarrubias OA, Fry WE (2002) Potato late blight management in the Toluca valley: field validation of Sim-Cast modified for cultivars with high field resistance. Plant Dis 86: 1163-1168 42

Grunwald NJ, Rubio-Covarrubias OA, Fry WE (2000) Potato late-blight management in the Toluca valley: forecasts and resistant cultivars. Plant Dis 84: 410-416

Hämäläinen JH, Watanabe KN, Valkonen JPT, A. Arihara A, Plaisted RL, Pehu E, Miller L, Slack SA (1997) Mapping and marker-assisted selection for a gene for extreme resistance to potato virus Y. Theor Appl Genet 94: 192-197

Hawkes JG (1990) The Potato, evolution, biodiversity and genetic Resources, Belhaven Press, London

Helgeson JP, Pohlman JD, Austin S, Haberlach GT, Wielgus SM, Ronis D, Zambolim L, Tooley P, McGrath JM, James RV, Stevenson WR (1998) Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theor. Appl. Genet. 96: 738-742

Hermsen JGTh, Ramanna MS (1973) Double-bridge hybrids of *Solanum bulbocastanum* and cultivars of *Solanum tuberosum*. Euphytica 22: 457-466

Hosaka K, Ogihara Y, Matsubayashi M, Tsunewaki K (1984). Phylogenetic relationship between the tuberous *Solanum* species as revealed by restriction endonuclease analysis of chloroplast DNA. Jap J Genet 59: 349-369

Howard HW (1970) The genetics of the potato *Solarium tuberosum*. Logos Press, London

Huang S, van der Vossen EAG, Kuang H, Vleeshouwers VGAA, Zhang N, Borm TJA, van Eck HJ, Baker B, Jacobsen E, Visser RGF (2005) Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. Plant J 42: 251-261

Huang S, Vleeshouwers VGAA, Werij JS, Hutten RCB, van Eck HJ, Visser RGF, Jacobsen E (2004) The R3 resistance to *Phytophthora infestans* in potato is conferred by two closely linked R genes with distinct specificities. Mol Plant Microbe Interact 17: 428-435

Hutten RCB, van Berloo R (2001) An online potato pedigree database. URL: http://www.dpw.wau.nl/pv/query.asp

Jackson SA, Hanneman REJr (1999) Crossability between cultivated and wild tuber-and non-tuber-bearing *Solanums.* Euphytica 109: 51-67

Jacobs JME, van Eck HJ, Horsman K, Arens PFP, Verkerk-Bakker B, Jacobsen E, Pereira A, Stiekema WJ (1996) Mapping of resistance to the potato cyst nematode *Globodera rostochiensis* from the wild potato species *Solanum vernei*. Mol Breed 2: 51-60

Janssen GJW, van Norel A, Verkerk-Bakker B , Janssen R (1995) Resistance to *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* in wild tuber-bearing *Solanum* spp. Euphytica 92: 287-294

Kardolus JP (1998) A biosystematic analysis of *Solanum acaule*. PhD thesis. Wageningen Agricultural University, Wageningen, The Netherlands

Kardolus JP, van Eck HJ, van den Berg RG (1998) The potential of AFLPs in biosystematics, a first application in *Solanum* taxonomy (*Solanaceae*). Plant Syst Evol 210: 87-103

Kasai K, Morikawa Y, Sorri VA, Valkonen JPT, Gebhardt C, Watanabe KN (2000) Development of SCAR markers to the PVY resistance gene $Ry_{adg}$ based on a common feature of plant disease resistance genes. Genome 43: 1-8

Kreike CM, de Koning JRA, Vinke JH, van Ooijen JW, Gebhardt C, Stiekema WJ (1993) Mapping of loci involved in quantitatively inherited resistance to the potato cyst-nematode *Globodera rostochiensis* pathotype Ro1. Theor Appl Genet 87: 464-470

Kruijt M, Kip DJ, Joosten MHAJ, Brandwagt BF, de Wit PJGM (2005) The *Cf-4* and *Cf-9* resistance genes against Cladosporium fulvum are conserved in wild tomato species. Mol Plant Microbe Interact 18: 1011-1021

Kuang H, Woo SS, Meyers BC, Nevo E, Michelmore RW (2004) Multiple genetic processes result in heterogeneous rates of evolution within the major cluster disease resistance genes in lettuce. Plant Cell 16: 2870-2894

Kuhl J, Hanneman R, Havey M (2001) Characterization and mapping of *Rpi1*, a late-blight resistance locus from diploid (1EBN) Mexican *Solanum pinnatisectum* Mol Genet Genomics 265: 977-985

Leister D (2004) Tandem and segmental gene duplication and recombination in the evolution of plant disease resistance genes. Trends Genet 20:116-122

Leonards-Schippers C, Gieffers W, Gebhardt C, Salamini F (1992) The *R1* gene conferring racespecific resistance to *Phytophthora infestans* in potato is located on potato chromosome V. Mol Gen Genet 233: 278-283

Li X, van Eck HJ, Rouppe van der Voort JNAM, Huigen DJ, Stam P, Jacobsen E (1998) Autotetraploids and genetic mapping using common AFLP markers: the *R2* allele conferring resistance to *Phytophthora infestans* mapped on potato chromosome 4. Theor Appl Genet 96: 1121-1128

Love, SL (1999) Founding clones, major contributing ancestors, and exotic progenitors of prominent North American potato cultivars. Am. J. Potato Res 76: 263-272

Malcolmson JF, Black W (1966) New R genes in *Solanum demissum* Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary. Euphytica 15: 199-203

Malosetti M, van der Linden CG, Vosman B, van Eeuwijk FA (2007) A mixed-model approach to association mapping using pedigree information with an illustration of resistance to *Phytophthora infestans* in potato. Genet 175: 879-889

Marczewski W, Flis B, Syller J, Schäfer-Pregl R, Gebhardt C (2001) A major quantitative trait locus for resistance to Potato leafroll virus is located in a resistance hotspot on potato chromosome XI and is tightly linked to N-gene-like markers. Mol Plant-Microbe Interact 14: 1420-1425

Marczewski W, Flis B, Syller J, Strzelczyk-Zyta D, Hennig J, Gebhardt C (2004) Two allelic or tightly linked genetic factors at the PLRV.4 locus on potato chromosome XI control resistance to potato leafroll virus accumulation. Theor Appl Genet 109: 1604-1609

Matsubayashi M (1955) Studies on the species differentiation of the section *Tuberarium* of *Solanum III.* Behavior of meiotic chromosomes in F1 hybrid between *S. longipedicellatum* and *S. schickii* in relation to its parent species. Science Reports of the Hyogo University of Agriculture 2: 25-31

Matsubayashi M (1991) Phylogenetic relationships in the potato and its related species. In: Tsuchiya T, Gupta PK (eds) Chromosome engineering in plants: genetics, breeding, evolution. Part B. Elsevier, Amsterdam, the Netherlands, pp 93-118

Mauricio R, Stahl EA, Korves T, Tian D, Kreitman M, Bergelson J. (2003) Natural selection for polymorphism in the disease resistance gene *Rps2* of Arabidopsis thaliana. Genet 163: 735-746

McKee RK (1962) Identification of R-genes in *Solanum stoloniferum*. Euphytica 11: 42-46

Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW (2003) Genome-wide analysis of NBS-LRR-encoding Genes in *Arabidopsis*. Plant Cell 15: 809-834

Meyers BC, Morgante M, Michelmore RW (2002) TIR-X and TIR-NBS proteins: two new families related to disease resistance TIR-NBS-LRR proteins encoded in *Arabidopsis* and other plant genomes. Plant J 32: 77-92

Meyer RC, Milbourne D, Hackett CA, Bradshaw JE, McNichol JW, Waugh R (1998) Linkage analysis in tetraploid potato and association of markers with quantitative resistance to late blight (*Phytophthora infestans*). Mol Gen Genet 259: 150-160

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8: 1113-1130

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers

in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88: 9828-9832

Miller JT, Spooner DM (1999) Collapse of species boundaries in the wild potato *Solanum brevicaule* complex *(Solanaceae, S.* sect. *Petota):* molecular data. Plant Syst Evol 214: 103-130

Milligan SB, Bodeau J, Yaghoobi J, Kaloshian I, Zabel P, Williamson VM (1998) The root-knot nematode resistance gene Mi from tomato is a member of the leucine zipper nucleotide binding leucine-rich repeat family of plant genes. Plant Cell 10: 1307-1319

Monosi B, Wisser R J, Pennill L, Hulbert SH (2004) Full-genome analysis of resistance gene homologues in rice. Theor Appl Genet 109: 1434-1447

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497

Naess SK, Bradeen JM, Wielgus SM, Haberlach GT, McGrath JM, Helgeson JP (2000) Resistance to late blight in Solanum bulbocastanum is mapped to chromosome 8. Theor Appl Genet 101: 697-704

Oberhagemann P, Chatot-Balandras C, Schäfer-Pregl R, Wegener D, Palomino C, Salamini F, Bonnel E, Gebhardt C (1999) A genetic analysis of quantitative resistance to late blight in potato: towards marker-assisted selection. Mol Breed 5: 399-415

Pan Q, Liu YS, Budai-Hadrian O, Sela M, Carmel-Goren L, Zamir D, Robert Fluhr (2000) comparative genetics of nucleotide binding site-leucine rich repeat resistance gene homologues in the genomes of two dicotyledons: tomato and arabidopsis. Genetics. 155: 309-322

Park TH (2005) Identification, characterization and high-resolution mapping of resistance genes to *Phytophthora infestans* in potato. PhD thesis, Agricultural University, Wageningen, The Netherlands

Park TH, Gros J, Sikkema A, Vleeshouwers VG, Muskens M, Allefs S, Jacobsen E, Visser RG, van der Vossen EA (2005) The late blight resistance locus *Rpi-bib3* from *Solanum bulbocastanum* belongs to a major late blight R gene cluster on chromosome 4 of potato. Mol Plant Microbe Interact 18: 722-729

Park TH, Vleeshouwers VGAA, Hutten RCB, van Eck HJ, van der Vossen E, Jacobsen E, Visser RGF (2005) High-resolution mapping and analysis of the resistance locus *Rpi-abpt* against *Phytophthora infestans* in potato. Mol Breed 16: 33-43

Pineda O, Bonierbale MW, Plaisted RL, Brodie BB, Tanksley SD (1993) Identification of RFLP markers linked to the H1 gene conferring resistance to the potato cyst nematode *Globodera rostochiensis.* Genome 36: 152-156

Raker CM, Spooner DM (2002) Chilean tetraploid cultivated potato, *Solanum tuberosum*, is distinct from the Andean populations: microsatellite data. Crop Sci 42: 1451-1458

Rauscher GM, Smart CD, Simko I, Bonierbale M, Mayton H, Greenland A., Fry WE (2006) Characterization and mapping of *Rpi-ber*, a novel potato late blight resistance gene from *Solanum berthaultii*. Theor Appl Genet 112: 674-687

Reeves JC, Chiapparino E, Donini P, Ganal M, Guiard J, Hamrit S, Heckenberger M, Huang XQ, Van Kaauwen M, Kochieva E, Koebner R, Law JR, Lea V, Le Clerc V, van der Lee T, Leigh F, van der Linden G, Malysheva L, Melchinger AE, Orford S, Reif JC, Röder M, Schulman A, Vosman B, Van der Wiel C, Wolf M, Zhang D (2004) Changes over time in the genetic diversity of four major European crops - a report from the Gediflux Framework 5 project. In: Proc XVII EUCARPIA General Congress, Genetic Variation for Plant Breeding (Vollmann J, Grausgruber H, and Ruckenbauer P, eds). Tulln, Austria. pp. 3-8

Richly E, Kurth J, Leister D (2002) Mode of amplification and reorganization of resistance genes during recent arabidopsis thaliana evolution. Mol Biol Evol 19:76-84

Rieseberg LH (1996) Homology among RAPD fragments in interspecific comparisons. Mol Ecology 5: 99-105

Ritter E, Debener T, Barone A, Salamini F, Gebhardt C (1991) RFLP mapping on potato chromosomes of two genes controlling extreme resistance to potato virus X (PVX). Mol Gen Genet 227: 81-85

Rodriguez A and Spooner DM (1997) Chloroplast DNA analysis of *Solanum bulbocastanum* and *S. cardiophyllum*, and evidence for the distinctiveness of *S. cardiophyllum* Subsp. *ehrenbergii* (Sect. *Petota*). Systematic Botany 22: 31-43

Rohlf FJ (1992) NTSYS-pc, numerical taxonomy and multivariate system. Exeter Publishing, Ltd, New York

Rose LE, Bittner-Eddy PD, Langley CH, Holub EB, Michelmore RW, Beynon JL. (2004) The maintenance of extreme amino acid diversity at the disease resistance gene, RPP13, in Arabidopsis thaliana. Genetics 166: 1517-1527

Ross H (1979) Wild species and primitive cultivars as ancestors of potato varieties. Proceedings conference broadening genetic base of Crops. Pudoc, Wageningen. pp 237-245

Ross H (1986) Potato breeding: problems and perspectives. Advances in plant breeding 13. Paul Parey Verlag, Berlin, Germany

Rouppe van der Voort J, Lindeman W, Folkertsma R, Hutten RCB, Overmars H, van der Vossen E, Jacobsen E, Bakker J (1998) A QTL for broad-spectrum resistance to cyst nematode species (*Globodera spp*.) maps to a resistance gene cluster in potato. Theor. Appl. Genet 96: 654-661

Rouppe van der Voort J, Wolters P, Folkertsma R, Hutten R, van Zandvoort P, Vinke H, Kanyuka K,Bendahmane A, Jacobsen E, Janssen R, Bakker J (1997b) Mapping of the cyst nematode resistance locus Gpa2 in potato using a strategy based on comigrating AFLP markers. Theor Appl Genet 95: 874-880

Sanchez GM, Smart CD, Simko I, Bonierbale M, Ewing EE, May G, Greenland A, Fry WE (2000) Identification of two new R-genes to *Phytophthora infestans* from *Solanum berthaultii.* Phytopathology 90:68

Sandbrink JM, Colon LT, Wolters PJCC, Stiekema WJ (2000) Two related genotypes of *Solanum microdontum* carry different segregating alleles for field resistance to *Phytophthora infestans*. Mol Breed 6: 215-225

Scanchez MJ and Bradeen JM (2006) Towards efficient isolation of R gene orthogos form multiple genotypes: optimization of long range-PCR. Mol Breed 17: 137-148

Sliwka J, Jakuczun H, Lebecka R, Marczewski W, Gebhardt C, Zimnoch-Guzowska E (2006) The novel, major locus *Rpi-phu1* for late blight resistance maps to potato chromosome IX and is not correlated with long vegetation period. Theor Appl Genet 113: 685-695

Smilde WD, Brigneti G, Jagger L, Perkins S, Jones JDG (2005) Solanum mochiquense chromosome IX carries a novel late blight resistance gene Rpi-moc1. Theor Appl Genet 110: 252-258

Stahl EA, Dwyer G, Mauricio R, Kreitman M, Bergelson J (1999) Dynamics of disease resistance polymorphism at the *Rpm1* locus of *Arabidopsis* Nature 400: 667-671

Stewart CNJ, Via LE (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques. 14: 748-750

Sokal RR (1986) Phenetic taxonomy, theory and methods. Annu Rev Ecol Syst 17: 423-442

Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, Helgeson JP, Jiang J (2003) Gene RB cloned from *Solanumbulbocastanum* confers broad spectrum resistance to potato late blight. Proc Natl Acad Sci 100: 9128-9133

Sonnewald U, Hajirezaei M-R, Biemelt S (2003) Designer tubers for production of novel compounds. Proc BCPC Int Cong Crop Science and Technology I pp 123-132

Sorri VA, Watanabe KN, Valkonen JPT (1999) Predicted kinase-3a motif of a resistance gene analogue as a unique marker for virus resistance. Theor Appl Genet 99: 164-170

Spooner DM, Castillo RT (1997) Reexamination of series relationships of South American wild potatoes (*Solanaceae, Solanum S.* sect. *Petota*), evidence from chloroplast DNA restriction site variation. Am J Bot 84: 671-685

Spooner DM, Hijmans RJ (2001) Potato systematics and germplasm collecting, 1989-2000. Am J Potato Res 78: 237-268

Spooner DM, Hosaka K (1992) RFLP analysis of the wild potato species, *Solanum acaule* Bitter *(Solanum* sect. *Petota).* Theor Appl Genet 84: 851-858

Spooner DM, Mclean K, Ramsay G, Waugh R, Bryan GJ (2005a) A single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. Proc Natl Acad Sci 102: 14694-14699

Spooner DM, Peralta IE, Knapp S (2005b) Comparison of AFLPs with other markers for pylogenetic inference in wild tomatoes [*Solanum* L. section *Lycopersicon* (Mill.)] Wettst. ]. Taxon 54: 43-61

Spooner DM, Salas A (2006) Structure, biosytematics, and genetic resources. In : Gopal J, Khurana SMP (eds), Handbook of potato production, improvement, and postharvest management. The Haworth Press, Binghamton, New York, pp.17

Spooner DM, van den Berg RG, Miller JT (2001) Species and series boundaries of *Solanum* series *Longipedicellata* (*Solanaceae*) and phenetically similar species in ser. *Demissa* and ser. *Tuberosa*: implications for a practical taxonomy of sect. *Petota*. Am J Bot 88: 113-130

Spooner DM, van den Berg RG, Rodrigues A, Bamberg J, Hijmans RJ, Lara-Cabrera, S (2004) Wild Potatoes (*Solanum* Section *Petota*) of North and Central America. Syst Bot Monogr 68: 1-209

Stewart CNJ, Via LE (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques. 14: 748-750

Sukhotu T, Kamijima O, Hosaka K (2004) Nuclear and chloroplast DNA differentiation in Andean potatoes. Genome 47: 46-56

Swofford DL (2001) PAUP* Phylogenetic analysis using parsimony* and other methods. version 4b10. Sinauer, Sunderland, Massachusetts

Syed NH, Sorensen AP, Antonise R, van de Wiel C, van der Linden CG, van't Westende W, Hooftman DAP, Nijs HCMD, Flavell AJ (2006) A detailed linkage map of lettuce based on SSAP, AFLP and NBS markers. Theor Appl Genet 112: 517-527

Tommiska TJ, Hämäläinen JH, Watanabe KN, Valkonen JPT (1998) Mapping of the gene $Nx_{phu}$ that controls hypersensitive resistance to potato virus X in *Solanum phureja* IvP35. Theor Appl Genet 96: 840-843

Toxopeus HJ (1964) Treasure-digging for blight resistance in potatoes. Euphytica 13: 206-222

Turkensteen LJ, Flier WG, Wanningen R, Mulder A (2000) Production, survival and infectivity of oospores of *Phytophthora infestans*. Plant Pathol 49:688-696

Umaerus V (1973) Background paper for the late blight project planning conference of CIP. Report of the late blight planning conference at Mexico (August 1973), CIP, Lima, Peru. pp 22-36

van den Berg RG, Bryan GJ, del Rio A, Spooner DM (2002) Reduction of species in the wild potato *Solanum* section *Petota* series *Longipedicellata*. AFLP, RAPD and chloroplast SSR data. Theor Appl Genet 105: 1109-1114

van der Linden CG, Wouters DCAE, Mihalka V, Kochieva EZ, Smulders MJM, Vosman B (2004) Efficient targeting of plant disease resistance loci using NBS profiling. Theor Appl Genet 109: 384-393

van der Linden CG, Smulders MJM, Vosman B (2005) Motif-directed profiling: a glance at molecular evolution. In: Bakker FT, Chatrou LW, Gravendeel B, Pelser PB (eds) Plant species-level systematics: new perspectives on pattern and process. Regnum Vegetabile 143. ARG Gantner Verlag, Ruggell; Koeltz, Koenigstein, pp 291-303

van der Vossen E, Sikkema A, Hekkert BL, Gros J, Stevens P, Muskens M, Wouters D, Pereira A, Stiekema W, Allefs S (2003) An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. Plant J 36: 867-882

van der Vossen E, Gros J, Sikkema A, Muskens M, Wouters D, Wolters P, Pereira A, Allefs S (2005) The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi–1* gene homolog conferring broad-spectrum late blight resistance in potato. Plant J 44: 208-222

Villamon FG, Spooner DM, Orrillo M, Mihovilovich E, Pérez W, Bonierbale M (2005) Late blight resistance linkages in a novel cross of the wild potato species *Solanum paucissectum* (series *Piurana*). Theor Appl Genet 111:1201-1214

Vleeshouwers VGAA, van Dooijeweert W, Keizer LCP, Sijpkes L, Govers F, Colon LT (1999)

A laboratory assay for *Phytophthora infestans* resistance in various *Solanum* species reflects the field situation. Eur J Plant Pathol 105: 241-250

Volkov RA, Komarova NY, Panchuk I.I, Hemleben V (2003) Molecular evolution of rDNA external transcribed spacer and phylogeny of sect. *Petota (*genus *Solanum).* Mol Phylogenet Evol 29: 187-202

Volkov RA, Zanke C, Panchuk II, Hemleben V (2001) Molecular evolution of 5S rDNA of *Solanum* species (sect. *Petota*), application for molecular phylogeny and breeding. Theor Appl Genet 103: 1273-1282

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP, A new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407-4414

Yoder AD, Irwin JA, Payseur BA (2001) Failure of the ILD to determine data combinability for slow loris phylogeny. Syst Biol 50: 408-424

Appendix 1 Varieties used in Chapter 3

| Code | Variety | Year of release |
|---|---|---|
| 1 | Accent | 1991 |
| 2 | Accord | 1996 |
| 3 | Achat | 1976 |
| 4 | Admiral | 1999 |
| 5 | Adora | 1991 |
| 6 | Adretta | 1975 |
| 7 | Agata | 1990 |
| 8 | Aiko | 1989 |
| 9 | Ailsa | 1984 |
| 10 | Ajax | 1977 |
| 11 | Ajiba | 1992 |
| 12 | Akira | 1998 |
| 13 | Aladdin | 1972 |
| 14 | Albas | 1993 |
| 15 | Albatros | 1996 |
| 16 | Alcmaria | 1970 |
| 17 | Allerfrüheste Gelbe | 1922 |
| 18 | Alpha | 1925 |
| 19 | Alwara | 1985 |
| 20 | Amadeus | 1993 |
| 21 | Amalfy | 1972 |
| 22 | Amaryl | 1963 |
| 23 | Ambiance | 1996 |
| 24 | Amex | 1972 |
| 25 | Amigo | 1967 |
| 26 | Aminca | 1973 |
| 27 | Amorosa | 2000 |
| 28 | Annabel | 1974 |
| 29 | Anosta | 1977 |
| 30 | Anya | 1996 |
| 31 | Aphrodite | 1990 |
| 32 | Apollo | 1970 |
| 33 | Aquila | 1942 |
| 34 | Ardenta | 1997 |
| 35 | Ari | 1957 |
| 36 | Arinda | 1993 |
| 37 | Aristo | 1959 |
| 38 | Arka | 1965 |
| 39 | Arkula | 1975 |
| 40 | Armada | 1996 |
| 41 | Arran Banner | 1927 |
| 42 | Arran Chief | 1911 |
| 43 | Arran Comet | 1956 |
| 44 | Arran Peak | 1935 |
| 45 | Arran Pilot | 1930 |
| 46 | Arran Victory | 1918 |
| 47 | Astoria | 1997 |
| 48 | Atica | 1971 |
| 49 | Atlantic | 1976 |
| 50 | Auk | 1992 |
| 51 | Aula | 1974 |
| 52 | Aurora | 1973 |
| 53 | Ausonia | 1984 |
| 54 | Avalanche | 1989 |
| 55 | Avanti | 1973 |
| 56 | Avenir | 1957 |
| 57 | Aziza | 1994 |
| 58 | Ballade | 1998 |
| 59 | Balmoral | 1989 |
| 60 | Barima | 1953 |
| 61 | Barna | 1993 |
| 62 | Belladona | 1997 |
| 63 | Benno Vrizo | 1994 |
| 64 | Berber | 1990 |
| 65 | Beteka | 1942 |
| 66 | Bevelander | 1925 |
| 67 | Bintje | 1910 |
| 68 | Blanka | 1972 |
| 69 | Blauwe Eigenheimer | 1907 |
| 70 | Bodenkraft | 1963 |
| 71 | Bolesta | 1995 |
| 72 | Bona | 1944 |
| 73 | Bonanza | 1993 |
| 74 | Bonaparte | 1987 |
| 75 | Bright | 1988 |
| 76 | British Queen | 1894 |
| 77 | Broca | 1969 |
| 78 | Burmania | 1957 |
| 79 | Caesar | 1994 |
| 80 | Calgary | 1989 |
| 81 | Calla | 1990 |
| 82 | Cantate | 1999 |
| 83 | Capella | 1943 |
| 84 | Cardinal | 1972 |
| 85 | Carina | 1972 |

Appendix 1 continued

| Code | Variety | Year of release |
|---|---|---|
| 86 | Carlingford | 1982 |
| 87 | Carlita | 1991 |
| 88 | Carmona | 2000 |
| 89 | Carola | 1979 |
| 90 | Casanova | 1997 |
| 91 | Catarina | 1966 |
| 92 | Catriona | 1920 |
| 93 | Celine | 1999 |
| 94 | Charisma | 1995 |
| 95 | Charles Downing | 1887 |
| 96 | Charlotte | 1981 |
| 97 | Christa | 1975 |
| 98 | Cicero | 1995 |
| 99 | Cilena | 1981 |
| 100 | Cinja | 1983 |
| 101 | Civa | 1960 |
| 102 | Claret | 1996 |
| 103 | Cleopatra | 1980 |
| 104 | Climax | 1955 |
| 105 | Clivia | 1962 |
| 106 | Colleen | 1993 |
| 107 | Colmo | 1975 |
| 108 | Columbus | 1996 |
| 109 | Compagnon | 1974 |
| 110 | Concorde | 1989 |
| 111 | Concurrent | 1985 |
| 112 | Condea | 1958 |
| 113 | Condor | 1937 |
| 114 | Conference | 1948 |
| 115 | Blue Congo | unknown |
| 116 | Cornado | 1994 |
| 117 | Cosima | 1959 |
| 118 | Cosmos | 1994 |
| 119 | Courage | 1998 |
| 120 | Craige Defiance | 1938 |
| 121 | Craige Royal | 1947 |
| 122 | Craigs Bounty | unknown |
| 123 | Red Craigs Royal | 1955 |
| 124 | Cupido | 1995 |
| 125 | Cycloon | 1996 |
| 126 | Dali | 1995 |
| 127 | Daresa | 1969 |
| 128 | Darwina | 1981 |
| 129 | Datura | 1957 |
| 130 | Depesche | 1942 |
| 131 | Désirée | 1962 |
| 132 | Deva | 1940 |
| 133 | Diamant | 1982 |
| 134 | Disco | 1993 |
| 135 | Ditta | 1989 |
| 136 | Divina | 1997 |
| 137 | Donald | 1996 |
| 138 | Donella | 1989 |
| 139 | Donetta | 1989 |
| 140 | Doon Star | 1926 |
| 141 | Dorado | unknown |
| 142 | Doré | 1947 |
| 143 | Dr McIntosh | 1944 |
| 144 | Draga | 1969 |
| 145 | Drayton | 1976 |
| 146 | Drop | 1998 |
| 147 | Dunbar Rover | 1936 |
| 148 | Dunbar Standard | 1936 |
| 149 | Dundrod | 1987 |
| 150 | Eba | 1966 |
| 151 | Eclipse | 1940 |
| 152 | Edzina | 1974 |
| 153 | Eersteling | 1900 |
| 154 | Rode Eersteling | 1925 |
| 155 | Ehud | 1967 |
| 156 | Element | 1969 |
| 157 | Elkana | 1978 |
| 158 | Elvira | 1981 |
| 159 | Empire | 1989 |
| 160 | Epicure | 1897 |
| 161 | Erdgold | 1928 |
| 162 | Erntestolz | 1976 |
| 163 | Escort | 1988 |
| 164 | Estima | 1973 |
| 165 | Evergood | 1900 |
| 166 | Exempla | 1997 |
| 167 | Exquisa | 1992 |
| 168 | Fabula | 1997 |
| 169 | Falke | 1943 |
| 170 | Fambo | 1985 |

Appendix 1 continued

| Code | Variety | Year of release |
|---|---|---|
| 171 | Fanal | 1968 |
| 172 | Fatima | 1970 |
| 173 | Favorita | 1974 |
| 174 | Felsina | 1992 |
| 175 | Feska | 1998 |
| 176 | Festien | 2000 |
| 177 | Fianna | 1986 |
| 178 | Filea | 1993 |
| 179 | Fina | 1955 |
| 180 | Florijn | 1996 |
| 181 | Florissant | 1994 |
| 182 | Fontane | 1999 |
| 183 | forelle | 1979 |
| 184 | Foremost | 1955 |
| 185 | Fresco | 1985 |
| 186 | Frieslander | 1990 |
| 187 | Frila | 1968 |
| 188 | Frisia | 1991 |
| 189 | Fruhbote | 1935 |
| 190 | Fruhmolle | 1934 |
| 191 | Furore | 1930 |
| 192 | Gelda | 1973 |
| 193 | Gineke | 1950 |
| 194 | Gitte | 1974 |
| 195 | Gladstone | 1932 |
| 196 | Gloria | 1937 |
| 197 | Golden Wonder | 1906 |
| 198 | Goya | 2000 |
| 199 | Grandifolia | 1973 |
| 200 | Granola | 1975 |
| 201 | Grata | 1955 |
| 202 | Great Scot | 1911 |
| 203 | Hansa | 1957 |
| 204 | Harmony | 1999 |
| 205 | Heather | 1993 |
| 206 | Heida | 1946 |
| 207 | Hela | 1964 |
| 208 | Helena | 1991 |
| 209 | Hercules | 1986 |
| 210 | Hermes | 1973 |
| 211 | Hertha | 1980 |
| 212 | Hochprozentige | 1946 |
| 213 | Home Guard | 1943 |
| 214 | Hydra | 1969 |
| 215 | Idole | 1989 |
| 216 | Impala | 1989 |
| 217 | Indira | 1981 |
| 218 | Industrie | 1900 |
| 219 | Inova | 1999 |
| 220 | International Kidney | 1879 |
| 221 | Irene | 1953 |
| 222 | Irmgard | 1960 |
| 223 | Isabell | 1974 |
| 224 | Isna | 1969 |
| 225 | Jaerla | 1969 |
| 226 | Jaqueline | 1996 |
| 227 | Jetta | 1967 |
| 228 | Jubel | 1908 |
| 229 | Junior | 1990 |
| 230 | Juvena | 1997 |
| 231 | Kanjer | 1996 |
| 232 | Karakter | 1996 |
| 233 | Karatop | 1990 |
| 234 | Kardent | 1996 |
| 235 | Karida | 1987 |
| 236 | Karlena | 1988 |
| 237 | Karnico | 1987 |
| 238 | Kartel | 1993 |
| 239 | Katahdin | 1932 |
| 240 | Katinka | 1998 |
| 241 | Kennebec | 1963 |
| 242 | Kerr's Pink | 1907 |
| 243 | Kestrel | 1992 |
| 244 | King edward | 1902 |
| 245 | Kingston | 1981 |
| 246 | Kondor | 1985 |
| 247 | Konsul | 1999 |
| 248 | Koopman's Blauwe | 1937 |
| 249 | Krometa | 1993 |
| 250 | Kronia | 1974 |
| 251 | Krostar | 1973 |
| 252 | Kuras | 1996 |
| 253 | Kuroda | 1998 |
| 254 | Kurola | 1996 |
| 255 | Lady Claire | 1996 |

Appendix 1 continued

| Code | Variety | Year of release |
|---|---|---|
| 256 | Lady Felicia | 1997 |
| 257 | Lady Florina | 2000 |
| 258 | Lady Rosetta | 1988 |
| 259 | Lekkerlander | 1965 |
| 260 | Leo | 1969 |
| 261 | Lerche | 1950 |
| 262 | leyla | 1988 |
| 263 | Libertas | 1946 |
| 264 | Likaria | 1986 |
| 265 | Linda | 1974 |
| 266 | Linzer Delikatess | 1975 |
| 267 | Linzer Rose | 1969 |
| 268 | Liu | 1988 |
| 269 | Lumper | 1806 |
| 270 | Luna | 1982 |
| 271 | Lux | 1971 |
| 272 | Lyra | 1987 |
| 273 | Majestic | 1911 |
| 274 | Manna | 1975 |
| 275 | Marabel | 1993 |
| 276 | Marfona | 1979 |
| 277 | Maris Anchor | 1971 |
| 278 | Maris Bard | 1972 |
| 279 | Maris Peer | 1962 |
| 280 | Maris Piper | 1963 |
| 281 | Markies | 1997 |
| 282 | Marlen | 2000 |
| 283 | Mascotte | 1998 |
| 284 | Maxine | 1994 |
| 285 | May Queen | 1980 |
| 286 | Meerlander | 1947 |
| 287 | Merkur | 1935 |
| 288 | Midas | 1997 |
| 289 | Minerva | 1989 |
| 290 | Miriam | 1988 |
| 291 | Mirka | 1970 |
| 292 | Monalisa | 1982 |
| 293 | Mondial | 1988 |
| 294 | Moni | 1972 |
| 295 | Monitor | 1973 |
| 296 | Montana | 1971 |
| 297 | Morene | 1985 |
| 298 | Multa | 1964 |
| 299 | Nadine | 1987 |
| 300 | Navan | 1987 |
| 301 | Nederlander | 1942 |
| 302 | Nicola | 1975 |
| 303 | Nika | 1995 |
| 304 | Nomade | 1997 |
| 305 | Norchip | 1968 |
| 306 | Novita | 1994 |
| 307 | Orion | 1943 |
| 308 | Oscar | 1995 |
| 309 | Ostbote | 1933 |
| 310 | Ottena | 2000 |
| 311 | Pallina | 1995 |
| 312 | Panda | 1986 |
| 313 | Patersons Victoria | 1856 |
| 314 | Patrones | 1959 |
| 315 | Penta | 1983 |
| 316 | Pentland Beauty | 1995 |
| 317 | Pentland Crown | 1959 |
| 318 | Pentland Hawk | 1967 |
| 319 | Pentland Ivory | 1967 |
| 320 | Pentland Marble | 1970 |
| 321 | pentland Squire | 1970 |
| 322 | Pimpernel | 1953 |
| 323 | Pink Fir Apple | 1850 |
| 324 | Pirol | 2000 |
| 325 | Ponto | 1984 |
| 326 | Populair | 1928 |
| 327 | Power | 1999 |
| 328 | Prevalent | 1966 |
| 329 | Prima | 1969 |
| 330 | Procura | 1973 |
| 331 | Prominent | 1968 |
| 332 | protea | 2000 |
| 333 | Proton | 1975 |
| 334 | Provento | 1994 |
| 335 | Provita | 1967 |
| 336 | Prudal | 1952 |
| 337 | Quarta | 1979 |
| 338 | Raisa | 1997 |
| 339 | Raja | 1994 |
| 340 | Rapido | 1994 |

Appendix 1 continued

| Code | Variety | Year of release |
|---|---|---|
| 341 | Rathlin | 1994 |
| 342 | Realist | 2000 |
| 343 | Rebbeca | 1984 |
| 344 | Rector | 1968 |
| 345 | Red King Edward | 1916 |
| 346 | Red Lasoda | 1952 |
| 347 | Red Pontiac | 1970 |
| 348 | Red Salad | 1999 |
| 349 | Redbad | 1974 |
| 350 | Redskin | 1934 |
| 351 | Redstar | 1998 |
| 352 | Reichskanzler | 1886 |
| 353 | Remarka | 1992 |
| 354 | Renate | 1993 |
| 355 | Renova | 1972 |
| 356 | Resonant | 1974 |
| 357 | Resy | 1968 |
| 358 | Rex | 1998 |
| 359 | Rheinhort | 1959 |
| 360 | Rikea | 1984 |
| 361 | Rita | 1988 |
| 362 | Robijn | 1926 |
| 363 | Rocket | 1987 |
| 364 | Rode Pipo | 1982 |
| 365 | Rode Star | 1909 |
| 366 | Romina | 1989 |
| 367 | Rooster | 1990 |
| 368 | Rosalie | 1971 |
| 369 | Rosara | 1990 |
| 370 | Roscor | 1994 |
| 371 | Roseval | 1950 |
| 372 | Roxy | 1995 |
| 373 | Royal Kidney | 1899 |
| 374 | Rubin | 1972 |
| 375 | Russet Burbank | 1908 |
| 376 | Rustica | 1988 |
| 377 | Safrane | 1991 |
| 378 | Sanira | 1992 |
| 379 | Santana | 1994 |
| 380 | Santé | 1983 |
| 381 | Saskia | 1946 |
| 382 | Satina | 1993 |
| 383 | Saxon | 1992 |
| 384 | Sebago | 1938 |
| 385 | Secura | 1985 |
| 386 | Selma | 1972 |
| 387 | Sempra | 1997 |
| 388 | Seresta | 1995 |
| 389 | Shannon | 1994 |
| 390 | Sharpes Express | 1900 |
| 391 | Shetland Black | unknown |
| 392 | Shula | 1986 |
| 393 | Sibu | 1993 |
| 394 | Sieglinde | 1935 |
| 395 | Sientje | 1938 |
| 396 | Sierra | 1991 |
| 397 | Simone | 1997 |
| 398 | Sirius | 1997 |
| 399 | Sjamero | 1996 |
| 400 | Slaney | 1993 |
| 401 | Solara | 1989 |
| 402 | Solide | 1997 |
| 403 | Sommergold | 1987 |
| 404 | Sommerstarke | 1965 |
| 405 | Sophytra | 2000 |
| 406 | Spey | 1996 |
| 407 | Spunta | 1969 |
| 408 | Starga | 2000 |
| 409 | Stormont Dawn | unknown |
| 410 | Stormont Enterprise | 1969 |
| 411 | Sunbeam | 1995 |
| 412 | Surprise | 1954 |
| 413 | Symfonia | 1995 |
| 414 | Taiga | 1973 |
| 415 | Tango | 2000 |
| 416 | Tanja | 1967 |
| 417 | Terra | 2001 |
| 418 | Thyra | 1965 |
| 419 | Tiffany | 1999 |
| 420 | Tomensa | 1989 |
| 421 | Toni | 1947 |
| 422 | Triumf | 1921 |
| 423 | Ulla | 1970 |
| 424 | Ulme | 2000 |
| 425 | Ulster Chieftain | 1938 |

Appendix 1 continued

| Code | Variety | Year of release |
|---|---|---|
| 426 | Ulster Concord | 1964 |
| 427 | Ulster Dale | 1950 |
| 428 | Ulster Ensign | 1946 |
| 429 | Ulster Glade | 1961 |
| 430 | Ulster Premier | 1945 |
| 431 | Ulster Prince | 1947 |
| 432 | Ulster Sceptre | 1962 |
| 433 | Ulster Supreme | 1935 |
| 434 | Ultimus | 1935 |
| 435 | Up to date | 1894 |
| 436 | Urgenta | 1953 |
| 437 | Van Gogh | 1989 |
| 438 | Vebesta | 1990 |
| 439 | Velox | 1994 |
| 440 | Venouska | 1987 |
| 441 | Vento | 1994 |
| 442 | Vera | 1943 |
| 443 | Victoria | 1997 |
| 444 | Vitesse | 1997 |
| 445 | Vivaks | 1973 |
| 446 | Vivaldi | 2000 |
| 447 | Vokal | 1974 |
| 448 | Voran | 1936 |
| 449 | Warinka | 1974 |
| 450 | White Lady | 1994 |
| 451 | Wilja | 1967 |
| 452 | Wilpo | 1939 |
| 453 | Woudster | 1960 |
| 454 | Xantia | 1998 |
| 455 | Yukon Gold | 1980 |
| 456 | Zeeburger | 1948 |

Appendix 2 Varieties repeated for *MseI*-NBS profiling

| Variety |
|---|
| Adora |
| Aziza |
| Bintje |
| Darwinia |
| Desiree |
| Escort |
| Florijn |
| Frila |
| Frisia |
| Kartel |
| Krometa |
| Kuras |
| Kurola |
| Lady Christl |
| Nomade |
| Patroness |
| Prevalent |
| Russet Burbank |
| Sante |
| Saturna |
| Shepody |
| Xantia |

# Summary

Potato (*Solanum tuberosum* L.) is a crop with a large secondary gene pool, which contains many important traits that can be exploited in breeding programs. As late blight is one of the biggest problems in potato growing areas, the crop needs a large number of applications of fungicides to be able to grow in north–western Europe. There is a strong focus on resistance breeding. This thesis describes the use of nucleotide binding site (NBS) profiling to study *Solanum* systematics and to identify resistance gene markers, which might be applied by the breeding companies. It also studies in depth the diversity and evolution of two late blight resistance (R) genes *Rpi-blb1* and *Rpi-blb2* in a wide range of *Solanum* species.

**Chapter 2** evaluates the potential of NBS profiling for phylogeny reconstruction in a set of over 100 genebank accessions, representing 47 tuber-bearing *Solanum* species. Results from NBS profiling are compared with those from amplified fragment length polymorphism (AFLP). Cladistic and phenetic analyses show that the two techniques deliver trees with a similar topology and resolution, indicating that NBS profiling can be an alternative for phylogeny reconstruction. No clear effects of targeting resistance genes are observed in the NBS profiling tree. Within the group of tuber-bearing *Solanum* species, 91% of the intraspecific fragments from the co-migrating bands have sequence identity higher than 95%, indicating that homoplasy is limited (**Chapter 3**).

**Chapter 3** presents the changes in genetic diversity at resistance gene loci in a set of 456 European potato cultivars during the last 70 - 80 years. The genetic diversity at these loci increased slightly, which most likely reflects the breeding efforts to introgress resistances from wild species into cultivated potato. Several candidate R-gene markers are identified by linking NBS profiling markers with pedigree and phenotypic data of the cultivars. As homoplasy in NBS profiling markers is low, the markers could also be linked to tuber-bearing *Solanum* species that had contributed the resistance marker to the set of cultivars. One of the markers identified is very likely introgressed from *Solanum vernei*, as indicated by the presence of the marker in *S. vernei* accessions and in cultivars that have *S. vernei* in their pedigree. The marker

also correlates to the resistance data from the cultivars involved.

**Chapter 4** describes the allele mining of two late blight R-genes, *Rpi-blb1* and *Rpi-blb2*, originally derived from *S. bulbocastanum*. It also analyzes the structure of the cluster that contains *Rpi-blb1*, by determining the presence or absence of the genes flanking *Rpi-blb1 (RGA1-blb* and *RGA3-blb).* A wide range of *Solanum* species was screened for the presence of *RGA1-blb* and it was found to be present and highly conserved not only in all the tested tuber-bearing *Solanum* species but also in the non-tuber-bearing species *S. etuberosum*, *S. fernandezianum* and *S. palustre*, suggesting that *RGA1-blb* was already present before the divergence of tuber-bearing and non-tuber-bearing *Solanum* species. The allele frequency of *RGA3-blb* is, however, much lower. Highly conserved *Rpi-blb1* (>99.5%) homologues are discovered not only in *S. bulbocastanum* but also in *Solanum stoloniferum*, a distinct tetraploid species from the series *Longipedicellata*. A number of dominant R-genes (*Rpi-sto1*, *Rpi-plt1*, *Rpi-pta1* and *Rpi-pta2*) are identified in several F1 populations, derived from the relevant late blight resistant parental genotypes harboring the *Rpi-blb1* homologue. Furthermore, *Rpi-sto1* and *Rpi-plt1* reside at the same position on chromosome VIII as *Rpi-blb1*. We propose that the above four genes share the same ancestry with *Rpi-blb1* from *S. bulbocastanum.* Segregation data also indicates that an additional unknown late blight resistance gene is present in three of the four segregating populations. In contrast to *Rpi-blb1*, *Rpi-blb2* is not detected in the examined set of material.

Allele frequency and allelic diversity of *Rpi-blb1* and *Rpi-blb2* is analyzed in accessions from *S. bulbocastanum* and the closely related species *S. cardiophyllum* (**Chapter 5**). Highly conserved *Rpi-blb1* alleles are found in 24 Mexican accessions, but not in material originating from Guatemala. Sequence analysis of a randomly selected set of genotypes reveals 19 *Rpi-blb1* haplotypes. Our results confirm that *Rpi-blbl* belongs to the type II class of resistance genes that evolve slowly (**Chapter 4 and 5**). Sequences of all putative susceptible *Rpi-blb1* are identical, suggesting that a single mutation event generates this allele. *Rpi-blb2* is present in only eight *S. bulbocastanum* accessions but not in other wild species examined. This, taken together with the fact that all the *Rpi-blb2* alleles examined are identical, suggests that *Rpi-blb2* has evolved recently (**Chapter 4 and 5**).

**Chapter 6** discusses findings obtained from this study in a context of systematics and evolution. The *Rpi-blb1* gene is originally discovered and cloned from *S. bulbocastanum*, a species that cannot be crossed with the cultivated potato *S. tuberosum* directly. Our study shows that functional homologs of *Rpi-blb1* are also present in *S. stoloniferum*, a species that can be crossed with cultivated potato directly. So, the *Rpi-sto1* gene from *S. stoloniferum* should be easier to introduce into cultivated potato than the *Rpi-blb1* gene from *S. bulbocastanum*. We anticipate that for other resistance genes present in primitive species, a similar situation may exist, i. e. homologs being also present in more advanced species that can be more easily used for breeding. Therefore, before starting a potato breeding program in a species that does not allow an immediate cross with cultivated potato, evaluation of directly crossable germplasm for the presence of that gene may speed up the breeding program and save valuable time and money.

# Samenvatting

Aardappel (*Solanum tuberosum* L.) is een gewas met een grote secundaire gene pool waarin vele belangrijke eigenschappen aanwezig zijn, die in veredelings-programma's gebruikt kunnen worden. De aardappelziekte, veroorzaakt door de oomyceet *Phytophthora infestans* (Mont.) de Bary, is een van de ernstigste problemen in aardappel productiegebieden. Het telen van aardappelen in noord-west Europa is alleen mogelijk als het gewas veelvuldig behandeld wordt met bestrijdingsmiddelen. Om daarin verandering te brengen is er veel aandacht voor resistentie veredeling. Dit proefschrift beschrijft het gebruik van 'nucleotide binding site (NBS) profiling' om de systematiek van het geslacht *Solanum* te bestuderen en om markers voor resistentie te identificeren, die door veredelingsbedrijven kunnen worden toegepast. Ook worden in detail de diversiteit en evolutie van twee *Phytophthora* resistentie genen (*Rpi-blb1* en *Rpi-blb2*) bestudeerd in een groot aantal *Solanum* soorten.

**Hoofdstuk 2** evalueert de mogelijkheid die NBS profiling biedt voor fylogenie reconstructie, gebruikmakend van een set van meer dan 100 genenbank accessies, die 47 knoldragende *Solanum* soorten vertegenwoordigen. De resultaten van NBS profiling worden vergeleken met die verkregen met 'amplified fragment length polymorphisms' (AFLP). Cladistische en fenetische analyses laten zien dat de twee technieken bomen opleveren met vergelijkbare topologie en resolutie, hetgeen er op wijst dat NBS profiling een alternatief kan zijn voor AFLP in fylogenie reconstructie. De NBS profiling boom vertoonde geen duidelijk effect van het zich richten op resistentie genen. Bij deze knoldragende *Solanum* soorten had 91% van de co-migrerende banden sequenties die meer dan 95% similariteit vertoonden, wat er op wijst dat homoplasie beperkt is.

**Hoofdstuk 3** presenteert de veranderingen in de genetische diversiteit van resistentie gen loci in een set van 456 Europese aardappel rassen gedurende de afgelopen 70 - 80 jaar. De genetische diversiteit op deze loci nam iets toe, vermoedelijk door de veredelingsactiviteiten waarbij resistenties vanuit wilde soorten in de gecultiveerde aardappel werden ingebracht. Verscheidene kandidaat R-genen werden geïdentificeerd door de NBS profiling markers te koppelen aan afstammings en fenotypische

gegevens van de rassen. Daar de homoplasie in NBS profiling markers laag was konden de markers ook gekoppeld worden aan de knoldragende *Solanum* soorten die de resistenties bijdroegen aan de rassen. Een van de geïdentificeerde markers is zeer waarschijnlijk afkomstig uit *Solanum vernei*, gezien de aanwezigheid van de marker in zowel *S. vernei* accessies als in rassen die *S. vernei* in hun stamboom hebben. De marker was ook gecorreleerd met de resistentie data van de betrokken rassen.

**Hoofdstuk 4** beschrijft 'allele mining' van twee *Phytophthora* R-genen, *Rpi-blb1* en *Rpi-blb2*, oorspronkelijk geïdentificeerd in *S. bulbocastanum*. Ook wordt de structuur van de cluster die *Rpi-blb1* bevat geanalyseerd, door de aan- en afwezigheid van de genen die *Rpi-blb1* flankeren *(RGA1-blb* and *RGA3-blb)* vast te stellen. Een groot aantal *Solanum* soorten werd getest op de aanwezigheid van *RGA1-blb* en dit gen bleek aanwezig en sterk geconserveerd, niet alleen in alle geteste knoldragende *Solanum* soorten, maar ook in de niet-knoldragende soorten *S. etuberosum*, *S. fernandezianum* en *S. palustre*, hetgeen suggereert dat *RGA1-blb* reeds aanwezig was voor de divergentie van knoldragende en niet-knoldragende *Solanum* soorten. De allel-frequentie van *RGA3-blb* was echter veel lager. Sterk geconserveerde *Rpi-blb1* (>99.5%) homologen werden niet alleen in *S. bulbocastanum* aangetroffen maar ook in *S. stoloniferum*, een tetraploide soort uit de serie *Longipedicellata*. Een aantal dominante R-genen (*Rpi-sto1*, *Rpi-plt1*, *Rpi-pta1* en *Rpi-pta2*) werd geïdentificeerd in F1 populaties, welke gebaseerd waren op resistente genotypen die de *Rpi-blb1* homoloog bevatten. *Rpi-sto1* en *Rpi-plt1* blijken op dezelfde positie op chromosoom VIII te liggen als *Rpi-blb1*. De vier genoemde genen delen hun afkomst met *Rpi-blb1* uit *S. bulbocastanum.* Gegevens over uitsplitsing geven ook aan dat er een additioneel *Phytophthora* resistentie gen aanwezig is in drie van de vier uitsplitsende populaties. Anders dan *Rpi-blb1* werd *Rpi-blb2* niet aangetroffen in het onderzochte materiaal.

De allel-frequentie en de allelische diversiteit van *Rpi-blb1* en *Rpi-blb2* werd onderzocht in accessies van *S. bulbocastanum* en de nauw verwante soort *S. cardiophyllum* (**Hoofdstuk 5**). Sterk geconserveerde *Rpi-blb1* allelen werden aangetroffen in 24 Mexicaanse accessies, maar niet in materiaal afkomstig uit Guatemala. Met sequentieanalyse van een set genotypen werden 19 *Rpi-blb1* haplotypen ontdekt. De resultaten bevestigen dat *Rpi-blbl* behoort tot de klasse van type II resistentiegenen, die langzaam evolueren (**Hoofdstuk 4 and 5**). Alle

vermoedelijk vatbare *Rpi-blb1* sequenties zijn identiek, hetgeen suggereert dat dit allel door slechts één mutatie-gebeurtenis is ontstaan. *Rpi-blb2* is aanwezig in slechts acht accessies van *S. bulbocastanum* en niet in de andere onderzochte wilde soorten. Samen met het feit dat alle onderzochte *Rpi-blb2* allelen identiek zijn, suggereert dit dat *Rpi-blb2* recentelijk is geëvolueerd (**Hoofdstuk 4 and 5**).

**Hoofdstuk 6** bespreekt de uitkomsten van dit onderzoek in de context van systematiek en evolutie. Het *Rpi-blb1* gen werd oorspronkelijk ontdekt in en gekloneerd uit *S. bulbocastanum*, een soort die niet direct kruisbaar is met de cultuuraardappel. Dit onderzoek heeft aangetoond dat functionele homologen van *Rpi-blb1* ook aanwezig zijn in *S. stoloniferum*, een soort die wel direct kruisbaar is met de cultuuraardappel. Het *Rpi-sto1* gen uit *S. stoloniferum* zou makkelijker in de cultuuraardappel geïntroduceerd moeten kunnen worden dan het *Rpi-blb1* gen uit *S. bulbocastanum*. Het is te voorzien dat dit ook kan gelden voor andere resistentie-genen die aanwezig zijn in primitieve soorten, n.l. dat homologen aanwezig zijn in meer afgeleide soorten die makkelijker in de veredeling kunnen worden gebruikt. Het zal daarom nuttig zijn om, voor een aardappel veredelingsprogramma wordt gestart in een soort die niet direct kruisbaar is met de cultuuraardappel, direct kruisbaar germplasm te evalueren op de aanwezigheid van dat gen, zodat het veredelingsprogramma kan worden versneld, waardoor tijd en geld kan worden bespaard.

# 中文摘要

马铃薯是有巨大的次生基因资源的作物，资源中有很多重要的性状能用于马铃薯育种。晚疫病是马铃薯种植区最大的问题之一，在北欧和西欧国家，马铃薯生长中使用大量的药物，因此，抗病育种是马铃薯育种的重点。该论文用核苷酸结合位点图谱研究了茄属的系统发育和抗病基因标记的发掘，这些标记有可能被育种公司应用。论文还利用很多茄种资源详细研究了两个晚疫病抗病基因 *Rpi-blb1* 和 *Rpi-blb2* 的多态性和进化。

第二章利用 100 多份基因库资源 47 个种，评估了核苷酸结合位点图谱在系统发育重建的可能性。比较分支系统和表型分析结果，核苷酸结合位点图谱和扩增片断长度多态性图谱产生相似的系统发育树，表明核苷酸结合位点图谱能够用于系统发育重建。核苷酸结合位点图谱系统发育树不能明显区分出抗病基因资源和非抗病基因资源。马铃薯种的共分离片断序列分析表明，91%的共分离片断同源性在 95%以上，说明核苷酸结合位点图谱非同源相似程度低（第三章）。

以过去七八十年中456个欧洲马铃薯品种为材料，第三章报告了抗病基因位点遗传多态性的变化。遗传多态性轻微增加，这在很大程度上是因为野生的马铃薯种被用于了马铃薯的育种。通过结合核苷酸结合位点图谱标记和品种的系谱及表型分析发现了几个候选抗性基因标记。由于核苷酸结合位点图谱的低非同源相似程度，这些标记也被用于马铃薯种和品种之间的比较，其中，这些马铃薯种贡献了抗病基因标记给马铃薯品种。一个标记有可能来自种*Solanum vernei* ，因为此标记既存在于*S. vernei* 资源中，也存在于系谱中有*S. vernei*的马铃薯品种中。 此标记的存在也与品种的表型数据相关。

第四章描述了两个晚疫病抗病基因 *Rpi-blb1* and *Rpi-blb2* 的等位基因发掘。这两个抗病基因起源于 *S. bulbocastanum* 种。通过判断 *Rpi-blb1* 侧翼区 *RGA1-blb* and *RGA3-blb* 的存在与否，第四章还分析了 *Rpi-blb1* 基因家族的结构。*RGA1-blb* 序列高度同源，不仅存在于所有研究的结薯马铃薯种中而且还存在于非结薯马铃薯种 *S. etuberosum*, *S. fernandezianum* 和 *S. palustre*，这些表明 *RGA1-blb* 在结薯马

铃薯种和非结薯马铃薯种的分化之前就已经存在。相对而言，*RGA3-blb* 的位点发生频率较低。在两个种 *S. bulbocastanum* 和 *Solanum stoloniferum* 中发现了高度同源的 *Rpi-blb1* (>99.5%)。*Solanum stoloniferum* 是属于分类单元 series *Longipedicellata* 中的一个四倍体种。在几个以含有 *Rpi-blb1* 同源类似物抗病植株为亲本的 F1 群体中发现了几个显性抗病基因 *Rpi-sto1*, *Rpi-plt1*, *Rpi-pta1* and *Rpi-pta2*。另外，与 *Rpi-blb1* 相同，*Rpi-sto1* 和 *Rpi-plt1* 也位于 8 号染色体。 我们认为，以上四个基因和来自于 *S. bulbocastanum* 的 *Rpi-blb1* 有共同的祖先。分离数据也表明，在三个分离群体中各含有另外一个未知的晚疫病抗病基因。和 *Rpi-blb1* 不同的是，在所有研究的材料中都没有发现 *Rpi-blb2* 等位基因。

第五章分析了 *Rpi-blb1* 和 *Rpi-blb2* 在种 *S. bulbocastan* 和近缘种 S. cardiophyllum 的发生频率和多态性。在 24 份墨西哥资源中发现了高度保守的 *Rpi-blb1* 等位基因，在来自危地马拉的资源中没有发现 *Rpi-blb1* 等位基因。随机选取 *Rpi-blb1* 等位基因，序列分析识别到了 19 个单元型， *Rpi-blbl* 属于进化慢的 II 型抗病基因 (第四章和第五章)。 所有推定的感病 *Rpi-blb1* 序列均相同，意味着单个突变事件产生了这个感病位点。只是在 8 份 *S. bulbocastanum* 资源中发现了 *Rpi-blb2* 等位基因，并且所有的 *Rpi-blb2* 等位基因具有相同的序列。所有这些表明 *Rpi-blb2* 进化发生在不久以前 (第四章和第五章)。

第五章在系统发育和进化的基础上讨论了论文的研究结果。基因 *Rpi-blb1* 最先在种 *S. bulbocastanum* 中被发现和克隆，这个种不能直接用于与栽培马铃薯的杂交。论文的研究结果表明，有功能的 *Rpi-blb1* 等位基因也存在于能与栽培马铃薯直接杂交的种 *S. stoloniferum* 中。 因此，相对于来源于 *S. bulbocastanum* 的 *Rpi-blb1* 抗病基因，来源于 *S. stoloniferum* 的 *Rpi-blb1* 等位抗病基因 *Rpi-sto1* 能够较为容易转入栽培马铃薯中。我们预测在其他的原生种中其它抗病基因也有可能有类似情况，即：抗病基因同源类似物存在于相对较为高级的种中，而这些种又能比较容易的应用于育种。因此，在开始一个不能直接用于杂交育种的马铃薯种的育种项目时，研究该抗病基因在其他可直接用于杂交育种的种有可能加速育种进程，节省时间并降低费用。

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I would like to thank teachers in oil crop institute (CAAS) for their help during my study in Wuhan, especially Prof. Li Guangming, Hua Fang and all teachers in the soybean group. Thanks go to teachers in CAAS graduate school, especially Prof. Lu Qingguang, Yu Zirong, Wang Chunxia, Jiang Xiwen and Wen Yang. I will never forget the help from IVF, especially Prof. Wang Xiaowu, Liu Guangshu, Prof. Sun Rifei and Jin Liping, Zhang Yanguo, Yang Baojun, Kang Jungen, Li Ying and Liu Yanling. Teachers in International Co-operation Agency are acknowledged for the help on funding application.

Prof. Sui Qijun, thanks for providing the field for late blight screen in Yunnan. Xianping,

you were so kind to arrange my stay in Kunming. I benefited much from our co-operation. Unfortunately, due to the extremely dry weather in Kunming in 2005, our second year late blight field test failed. Therefore, one chapter which should have your name on the author list aborted. I feel sorry for this. I hope the materials I introduced to you will help your breeding program. Zhijian, we knew each other from the Sino-Dutch summit meeting in Beijing, 2003. Later, you kindly offered the *phytophthora* isolates to me for free. Without your help, my late blight screen in China would have been impossible. I am so excited that in 2006 you visited Netherlands, giving me the chance to show my gratitude. These pathogen experts should be mentioned: Prof. Li Baoju and Li Xixiang (IVF), Yang Yanli (Yunnan Agricultural University), Zhu Jiehua (Hebei Agricultural University). Thank you all for your help when I visited your labs.

Far away from home, many friends around make the time in Wageningen less alone: Liying, Yehong, Jianjun, Junming, Wu Jian, Aiguo, Guo Jun, Chengwei, Limei, Yongyan-CAAS PhDs in the Sino-Dutch program; Jifeng, Lou Ping, Ningwen, Yuling, Wan Xi, Guangcun, Zhang Lu, Zhongkui, Xingfeng, Xiaomin, Caiyun, Jiayou, Jing Qi; Zhihui, Zhongshan, xuekui, Danfeng, Shujun. Jack, I am grateful for the accommodation in Gouda. I also enjoyed very much the travel with you. Herman, thanks for the information about Paris which made my travel very safe.

Andrew and Wilmi, thanks for giving me a “home” in the Netherlands. Whenever I have trouble, I can’t help calling you or just dropping by without any appointment. So many memories are in my mind: You drove me to Ede hospital after I was bitten by the dog; you accompanied me to visit the dentist; Every Christmas I was in your house sharing dinner with you; I had wonderful time with your family members. Wilmi, I wish you good luck for your study.

I would like to thank teachers and friends in China. My master supervisor Prof. Du Guoqiang and your family (Shi Xiaoxin and your parents-in-law), master supervisor Prof. Ge Huibo, Zhang Xueying, Shi Yun, your trust, encouragement and help make me realized how wonderful the world is. Zhanglan, I am so lucky to have had you as classmate. We have known each other for 12 years. I am so happy that you got married during the May holiday this year. Wish you a very happy time. Guochao, 15 years passed since we first met. Glad to know that what I have done influenced your decisions again and again, which made two of us be together much longer. Thanks for listening to my complaints before my going abroad and after my going back to Beijing in 2003. Yifan, Mada and Wenzhen, distance is never a problem for you to reach me. You shared much more tears than laughs with me during my stay in the Netherlands, but none of you ever complained. Without your mental support, I would have never finished my PhD study.

During the PhD journey, I owe too much to my family. Grandma, you left me without me

by your side, but your love and spirit are always with me; I am in debt to my sisters and brothers-in-law. Thanks for taking care of our parents when I am not around. Mom and Dad, I can imagine how tough it is for you when I am far away for such a long time. Thank you for your love and support for so many years. I am back at home soon, so please don't worry.

Miqia Wang 王密恰

Wageningen, the Netherlands

May, 2007

# Curriculum Vitae

Miqia Wang was born on August 13, 1976, in Raoyang, Hebei Province, China. She started her bachelor study in Hebei Agricultural University in 1999. As a distinguished graduate, she commenced her master study in the same university without exam and obtained the MSc degree majored in horticulture in 2002. Afterwards, she entered Chinese Academy of Agricultural Sciences (CAAS) in Beijing as a PhD student. She finished the study (compulsory for the graduation of PhDs in China) in Beijing, CAAS. In the end of 2002, she got the chance to participate the Sino-Dutch PhD program. In early 2003, she worked on soybean in Oil Crop Research Institute in Wuhan, CAAS. She started to work in Plant Research International (PRI) in mid-April in 2003. After seven months, she went back to China working on late blight screen in the Sino-Dutch joint lab in Beijing. After obtaining two scholarships, she came to PRI in mid-April 2005. The project she worked in PRI ended up with the completion of this thesis.

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# Education Statement of the Graduate School

# Experimental Plant Sciences

**Issued to:** Miqia Wang
**Date:** 12 June 2007
**Group:** Biosystematics and Plant Research International
Wageningen University and Research Centre

| 1) Start-up phase | date |
|---|---|
| ► **First presentation of your project** | |
| Evolution of resistance gene analogs in *Solanum* | Aug 13, 2003 |
| ► **Writing or rewriting a project proposal** | |
| Utility of NBS profling on systematics, fellowship programme of the netherlands ministry of agriculture, nature management and food qualtity | Aug 13-23, 2004 |
| Evolution of late blight genes in Solanum, National abroad association funding of China | Dec 15-25, 2004 |
| ► **Writing a review or book chapter** | |
| ► **MSc courses** | |
| ► **Laboratory use of isotopes** | |
| Safe handling with radioactive materials and sources | May 19-23, 2003 |
| *Subtotal Start-up Phase* | *9.0 credits** |

| 2) Scientific Exposure | date |
|---|---|
| ► **EPS PhD student days** | |
| 2005 EPS PhD Day, Radboud University Nijmegen (Netherlands) | Jun 2, 2005 |
| 2006 French PhD Day, Paris (France) | Jun 9, 2006 |
| ► **EPS theme symposia** | |
| EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Leiden University (Netherlands) | Jun 23, 2005 |
| EPS theme 4 symposium 'Genome Plasticity', Wageningen University (Netherlands) | Dec 9, 2005 |
| ► **NWO Lunteren days and other National Platforms** | |
| NWO-ALW, Experimental Plant Sciences, Lunteren (Netherlands) | Apr 4-5, 2005 |
| ► **Seminars (series), workshops and symposia** | |
| Flying seminar: Discovery of funcitoanl elements in the Arabidopsis genome | Sep 26, 2005 |
| Flying seminar: small RNA, chormosome organization and gene expression | Oct 23, 2006 |
| Natural variation: genomics and evolution | Nov 16, 2006 |
| flying seminar: RNA silencing pathways-mechanisms and functions | Mar 26, 2007 |
| ► **Seminar plus** | |
| ► **International symposia and congresses** | |
| Plant genomics: from crop production to healthy food, Beijing (China) | Nov 9-11, 2003 |
| 5th Wold Potato Congress, Kunming (China) | Mar 24 - 30, 2004 |
| EU Bioexploit Project symposia, Amsterdam (Netherlands) | Apr 12, 2006 |
| ► **Presentations** | |
| Oral: Utility of NBS profiling on systematics, Wageningen (Netherlands) | Sep 9, 2003 |
| Oral & Poster: Systematic relationship based on resistance gene analogs in Solanum, Beijing (China) | Nov 9, 2003 |
| Oral presentation, late blight screen on Solanum species in Yunnan (China) | Apr 16, 2004 |
| Oral: Utility of NBS profiling for plant systematics, Wageningen (Netherlands) | Dce 9, 2005 |
| Oral: Allelic diversity for late blight resistance genes in tuber bearing *Solanum,* Amsterdam (Netherlands) | Apr 12, 2006 |
| Oral: Allelic diversity of *Rpi-blb1* and *Rpi-blb2* in S*olanum* , wageningen (Netherlands) | Sep 25, 2006 |
| ► **IAB interview** | Jun 21, 2005 |
| ► **Excursions** | |
| Tomato & potato late blight in China, Inst. Vegetable and Flowers (IVF) , CAAS, Beijing (China) | Nov 17-21, 2004 |
| *Subtotal Scientific Exposure* | *12.8 credits** |

| 3) In-Depth Studies | date |
|---|---|
| ► **EPS courses or other PhD courses** | |
| Molecular phylogenies: construction and interpretation, Wageningen (Netherlands) | Nov 4-7, 2003 |
| Advanced biochemistry, graduate school of CAAS (China) | Oct 7-11, 2002 |
| ► **Journal club** | |
| weekly literature discussion in IVF, CAAS or PRI (Netherlands and China) | 2003-2007 |
| ► **Individual research training** | |
| Overview of pathogen---*Phytophthora infestans,* Yunnan Acad. of Agric. Sciences, Yunnan Agricultural University, China | Dec 10-14, 2004 |
| *Subtotal In-Depth Studies* | *7.2 credits** |

| 4) Personal development | date |
|---|---|
| ► **Skill training courses** | |
| How to write scientific proposal, CAAS (China) | Nov 21-25, 2002 |
| PhD competence test (Netherlands) | Nov 8, 2005 |
| Endnote 9.0 introduction (Netherlands) | Oct 10, 2005 |
| PhD Scientic writing, CENTA language center, Wageningen (Netherlands) | Sep 26-Oct 14, 2006 |
| ► **Organisation of PhD students day, course or conference** | |
| Course on molecular techniques, Beijing (China) | Nov 10-14, 2004 |
| ► **Membership of Board, Committee or PhD council** | |
| *Subtotal Personal Development* | *5.4 credits** |

| **TOTAL NUMBER OF CREDIT POINTS*** | **34.4** |
|---|---|

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

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