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Applied Biotechnology to Combat Late Blight in Potato Caused by *Phytophthora Infestans*

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Abstract Potato is an important crop, grown worldwide. It suffers from many pests and diseases among which late blight, caused by the oomycete *Phytophthora infestans*, is the worst. The disease is still causing major damage in many potato production areas and control is only possible by applying fungicides frequently. The knowledge on the molecular biology and genetics of the interaction between the plant and the oomycete is developing rapidly. These are relevant fields of study, currently dominated by the discovery of many resistance genes and numerous effector proteins and the analysis of their specific mode of action. These studies may yield essential information needed for the development of durable resistance. The long-term and worldwide effort to breed for resistance so far has had little effect. A novel breeding approach may change this. It is based on cisgenic modification (CM) consisting of marker-free pyramiding of several resistance genes and their spatial and temporal deployment yielding dynamic varieties that contain potato genes only. It is envisioned that this CM approach with potato's own genes will not only prove societally acceptable but may also result in simplifications in the legislation on use of the CM approach. Various parties in the potato research arena intend to cooperate in this novel approach in a number of developing countries where potato substantially contributes to food security. The use of resources such as land, water and energy improves when the effect of late blight is markedly reduced.

Keywords Breeding strategy · Cisgenesis · Effectors · Late blight · *Phytophthora infestans* · *R* genes · Resistance management · *Solanum*

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Introduction

This paper intends to contribute to the debate on the exploitation of plant diversity for realizing a strong reduction in the use of fungicides in the production of potato by:

1. Providing an overview of trends in potato production, showing a shift of production and consumption of potato from Europe and North America to emerging economies, especially in Asia.
2. Analysing the societal costs of late blight, caused by *Phytophthora infestans*, both in developed countries and in developing countries.
3. Providing an update on the latest developments in the molecular biology of the interaction between the potato plant and the oomycete.
4. Describing a strategy to develop durable resistance to late blight in dynamic varieties.

The approach is to provide the information in a way that is also comprehensible for the layman.

Statistics and Trends in Potato Production and Consumption

Presently the potato is grown in almost all countries of the world. The only exceptions are countries near the equator that lack cool agro-ecological zones in the highlands. The total area in 2005 was almost 20 million hectares with a total world production of over 300 Mt (Table 1). After wheat (630 Mt) and rice (608 Mt) it is the third crop in order of importance consumed by men. Maize (725 Mt) is an important crop as well but much of this commodity is used as feed or for industrial processing (e.g., into bioethanol). By far the largest areas of potato are found in Asia and Europe (Table 1). As Asia has more than five times as many inhabitants as Europe the consumption per capita per year is by far the largest in Europe: almost three times the global average of 31 kg per person per year. Although potato production is rapidly increasing in Africa (data not shown), the consumption per capita in Africa is still the lowest of all continents. Observations per country rather than per continent (Table 2) show that China is the largest producer of potato with over 70 Mt produced

Table 1 Potato area, potato production, potato yield, population and potato consumption by region in 2005 (source: www.faostat.org)

Area	Area (× 1000 ha)	Production (1000 t)	Yield (t/ha)	Population (million)	Consumption (kg/capita)
Africa	1,541	16,707	10.8	904	13.9
Asia/Oceania	8,733	137,344	15.7	3,935	23.9
Europe	7,474	130,224	17.4	739	87.8
Latin America	964	15,683	16.3	562	20.7
North America	616	25,345	41.2	330	60.0
WORLD	19,327	323,302	16.8	6,485	31.3

Table 2 Top 10 producers of potato in 2007 (source www.faostat.org)

Country	Production (t)
China	72,040,000
Russian Federation	36,784,200
India	26,280,000
United States	20,373,267
Ukraine	19,102,300
Poland	11,791,072
Germany	11,643,769
Belarus	8,743,976
Netherlands	7,200,000
France	6,271,000

per year, almost a quarter of the annual global production. China is followed by the Russian Federation with about half of China's production level. The production of over 7 Mt of potato in the Netherlands, a small country in terms of acreage of arable farming, is due to the cropping frequency of potato: about 25% of the arable land in the country is under potato cultivation. The Netherlands exports about 70% of all potatoes it produces, notably as French fries and seed potatoes. With 700,000 tonnes of seed exported the country has an 80% share of the global seed potato export market.

Table 3 shows the top 10 countries where most potatoes and their products were consumed in total and the top 10 countries with the highest per capita consumption in 2005: China leads with almost 58 Mt consumed by the total population but does not appear in the top 10 of amount of potatoes consumed per capita. Similarly India with over 17 Mt produced—also due to the high number of inhabitants, like China surpassing one billion—does not appear in the top 10 potato consumers per capita. Note that the figures shown in Table 3 do not represent the amount of potatoes

Table 3 Top 10 potato consumers in quantity or consumption per capita in 2005 (source www.faostat.org)

Country	Quantity (t)	Country	Consumption (kg per capita)
China	57,594,193	Belarus	181
Russian Federation	18,828,000	Kyrgyzstan	143
India	17,380,730	Ukraine	136
United States	17,105,000	Russian Federation	131
Ukraine	6,380,850	Poland	131
United Kingdom	6,169,000	Rwanda	125
Germany	5,572,000	Lithuania	116
Poland	5,000,000	Latvia	114
Bangladesh	4,041,463	Kazakhstan	103
Iran	3,991,142	United Kingdom	102

produced in the country divided by the number of inhabitants (then the Netherlands would rank highest with 450 kg per capita) but it is the result of what is actually consumed (deducted from data on national production, what is exported, seed and rejected).

Considering the trends over the past decades (Fig. 1) it is striking that the area cropped with potato in the developing countries as they were classified in 1960 has increased from 3 Mha to well over 10 Mha (a three-fold increase) whereas the area in the developed countries (Japan, Australia, New Zealand, Europe including Russia, USA and Canada) halved from almost 20 Mha to less than 9 Mha at present. The reason for the increase in developing countries is that people there appreciate potatoes within their changing consumption patterns. There is a strong urbanization with a growth in quick service restaurants and supermarkets where processed potatoes are supplied. At the supply side, the potato crop also found a niche in rice-growing areas where potato fits in the cropping system as a winter crop. The development of agribusiness with a supply of fertilizers, pesticides and healthy seed and an organization to collect and process potatoes also greatly contributed to the strong increase in potato production in developing countries.

The main reason for the decline in the developed countries is that people eat less potato, especially table potato, but also less potato is used to feed pigs. The latter is the main reason for the decline of potato production in Eastern Europe. In Germany in 1960 the per capita potato production was 500 kg and in Poland even 1500 kg grown on about 2 Mha (much of it used as pig feed) whereas presently German annual per capita consumption levelled off at about 75 kg per person per year. In Poland it still is above 120 kg per person per year but also declining.

The development in the developing countries is best illustrated by the situation in China and India (Fig. 2). China produced in 1960 12 Mt of potato on 1.4 Mha with an average yield of 8.5 Mg ha^{-1}. In 2006, these figures were 70 Mt on 5.5 Mha with an average yield of almost 13 Mg ha^{-1}. Similarly, India in 1960 produced 3 Mt on 400,000 ha (7.5 Mg ha^{-1}) and in 2006 24 Mt on 1.4 Mha (17.1 Mg ha^{-1}). These two Asian countries showed that both area and yield can be increased simultaneously. In sub-Saharan Africa, however, the yields declined between 1994 and 2006 from 9 Mg ha^{-1} to 7.5 Mg ha^{-1}. The area increased 2.5 fold to 1.1 Mha producing 8 Mt in

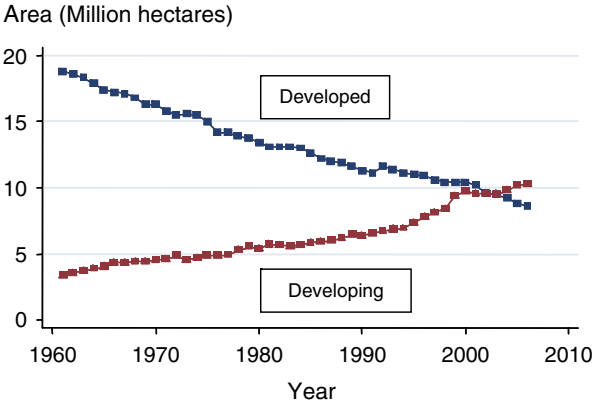

Fig. 1 Development of the global area cropped with potato from 1960 to 2008 (source: International Potato Center based on FAO data)

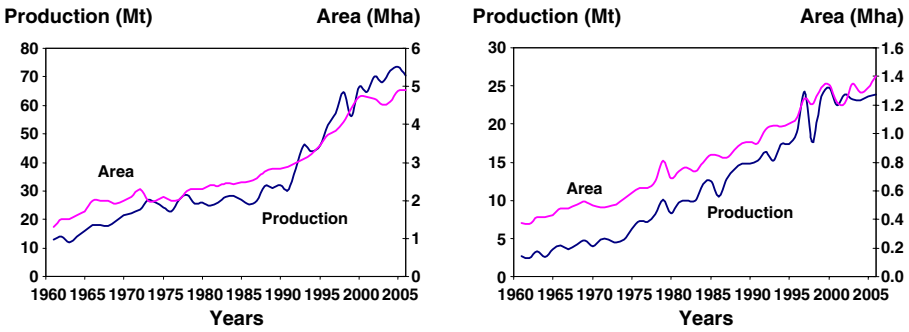

Fig. 2 Development of potato area and production in China (left) and India (right) since 1960 (source www.faostat.org)

2006. The main reasons for declining yields in sub-Saharan Africa are the lack of functioning seed potato schemes or systems and the unavailability of chemical fertilizers and pest control agents. And this is an important reason why late blight control is not only a highly relevant issue in Western agriculture but certainly also a matter of great importance in emerging economies.

Societal Costs of Late Blight Caused by *Phytophthora infestans*

Potato grows well in cool temperate climates as found in northern European summers, in subtropical winters, and in mountainous areas in the tropics. There are two main challenges to grow potatoes:

1. The vegetatively multiplied crop needs healthy seed—grown in an area or period of the year devoid of insects that transmit viruses—in order to be productive; and
2. The crop is not resistant to its main enemy: late blight caused by the oomycete *Phytophthora infestans*.

Over 400 years ago the potato was introduced to Europe by Spaniards and gradually replaced cereals in northern Europe. Linnaeus named the plant *Solanum tuberosum*. This introduction of potato was not accompanied by a simultaneous introduction of *Phytophthora infestans*. The oomycete arrived later when potato tubers infected with late blight originating from North America came to the European continent. In Ireland the crop had greatly contributed to increased population growth and therefore this country was hardest hit by the new disease. In a few years time in the 1840s potato yields were decimated because of the disease. This caused the Irish famine but is also linked to social turbulence on the European mainland (Zadoks 2008) and therefore the disease completely changed the societal landscape of the Old and of the New World.

Spores of late blight are carried by wind and, upon landing on a potato leaf, germinate and enter the leaf provided the leaves are wet from rain or dew for half a day. The disease spreads rapidly and can kill a crop entirely within a few weeks. If this happens early in the season yields may be reduced by up to 80%. The disease

can be controlled chemically by a weekly application of chemicals dissolved in water and sprayed over the canopy. In dry regions where crops are irrigated chemical control is somewhat less intensive. In developing countries where growers do not have access to chemicals yields tend to be about 25% of those in northern Europe or in the USA where yields roughly range from 40 Mg ha^{-1} to 60 Mg ha^{-1}. In the latter regions chemical control of late blight contributes to about 10–20% of the total production costs of the crop.

In order to estimate the economic importance of late blight in potato production, the Netherlands are taken as a case from which extrapolations are derived. For a previous report on this issue see also Haverkort et al. (2008). Much of the information described in this section is taken from that reference. In the Netherlands, the total area cropped with potato is 165,000 ha and the average yield is 45 Mg ha^{-1}. This results in a total production of 7.9 Mt which represents an average value of about M€ 790. Applying fungicides is associated with costs of the chemical applied and the costs of applying them (including costs for machines, labour, and energy). The number of applications varies between 10 and 16 per season. Seed potato crops receive fewer applications as they are harvested prematurely, but the fungicides used in seed production are more expensive. Growers tend to alternate systemic fungicides with contact fungicides to avoid build-up of resistance of the disease against the active ingredients. The total amount of fungicides applied on 165,000 ha of potato crop in the Netherlands is estimated to be 1,424 Mg. The costs of the fungicides are calculated at M€ 61.1 per year. The costs of applying on average 15 times per season (machinery, labour and fuel) are calculated at € 330 per hectare (M€ 54.4 national total). This means that costs of control (chemical + application) amount to M€ 115.5 for the country per year. Potato late blight not only leads to costs of control but also to costs due to losses. Financial losses caused by incidental premature harvest and immediate delivery to the market due to bad storability in ware potato are estimated to represent a value of M€ 1.4, assuming a frequency of once every 5 years on 10% of the acreage and resulting in a 5% loss of value. Spraying machines leave tracks in the fields and thereby cause local damage to the crop. This crop damage results in a yield loss of 3%. As crops are partly also sprayed with pesticides to control other pests and diseases (and this is especially true in the case of seed potato crops) a loss of 1% of the ware crops and starch crops and a loss of 0% of the seed crops can be attributed to the tracks created for the control of late blight. This represents a value of M€ 4.8. In the Netherlands organic production of seed potatoes takes place on 350 ha and production of ware potatoes on 1050 ha with average yields of 27 and 29 Mg ha^{-1} respectively, whereas conventional yields are 38 and 50 Mg ha^{-1} for seed and ware, respectively. In total, organic production on 1400 ha yields 25,900 t less than it would have in a conventional situation. Assuming that half of the yield loss is due to late blight and assuming a farm gate price of € 250/t leads to a loss of M€ 3.2. Adding up all losses incurred from late blight in the Netherlands leads to a figure of M€ 9.4. Added to the M€ 115.5 for control totals the costs of late blight in the Netherlands at M€ 124.9 which is 15.8% of total farm gate price.

Additional societal costs of potato production are associated with energy use and CO_2 emission when growing and harvesting the crop. The total amount of energy required to grow 1 ha of table potato from soil preparation through store loading is

25 GJ (de Wolf and van der Klooster 2006). This includes late blight control. Fifteen sprays to control late blight require about 30 l of diesel per ha. With 40 MJ per litre these 30 l of diesel per ha are equivalent to 1.2 GJ per ha. With 2.63 kg CO_2 produced when using 1 l of diesel, the 30 l of diesel per ha represent 79 kg CO_2 per ha. The energy content of the 10 kg fungicides applied per ha with 180 MJ per kg (Green 1987; Audsley et al. 1997) adds another 1.8 GJ per ha. The production of 1 kg of fungicide is associated with the production of 14.5 kg CO_2 (Green 1987; Audsley et al. 1997), thus 10 kg of fungicide per ha is equivalent to a carbon dioxide emission of 145 kg CO_2 per ha. In summary: *Phytophthora infestans* control in northern Europe is responsible for 3.0 GJ per ha of energy (12% of total production) and responsible for 224 kg per ha of CO_2 emission.

A first approximation of global costs and losses due to late blight may assume that late blight takes 16% of all 323 Mt of global potato. At 100 €/t the world potato production represents a value of € 32.3 billion. The 16% loss then represents an annual financial loss of € 5.2 billion per annum.

A further fine tuning of the annual global losses caused by late blight can be based on the following reasoning. The relative cost of controlling late blight on the entire potato area is roughly the same in the different developed countries, despite differences in conditions. It might be argued that in some areas—e.g., Idaho in the USA—the relative costs may be less than average as in these areas high yields are associated with relatively few applications. Similarly in other areas, e.g., in Argentina, yields are lower but frequency of application is comparable to that of the Netherlands so there the relative costs are somewhat higher. But overall these inaccuracies will balance out. The area with high yields associated with an adequate control of late blight is well over 0.6 Mha in North America and about 0.9 Mha in north-western Europe (UK, France, Netherlands, Belgium, Germany and Scandinavia), Japan and Oceania. These 1.5 Mha only represent about 7.5% of the global production area. Assuming a yield of 40 Mg ha^{-1}, the total production on these 1.5 Mha is 60 Mt representing a value of about € 6.0 billion. With 16% loss caused by *Phytophthora infestans*, financial losses equal € 1.0 billion.

About 92% of all potato crops grown worldwide have yields of approximately 15 Mg ha^{-1} as is inferred from calculations based on Table 1 data. Such yields are 25 Mg ha^{-1} below attainable yields (see yields in North America). If half of this amount is attributed to inadequate measures to control late blight (or to complete absence of control) and the remaining half to other yield-limiting and yield-reducing factors, then 0.92×20 Mha $\times 12.5$ Mg $ha^{-1} \times 100$ €/t equals a value of annual losses of € 23 billion. If we assume—conservatively—that only a quarter of the 25 Mg ha^{-1} yield gap is due to late blight the losses still become € 12 billion per annum. The areas with lowest yields that are mainly present in developing countries and previous eastern block countries suffer at least over € 10 billion per annum whereas growers in developed countries with high yields (7.5% of global potato production) suffer damage in the order of € 1 billion per year.

Hence, a late-blight free potato would especially impact people's food security and income in developing countries where losses would become much smaller and where the acreage of the potato crop would increase even more rapidly than it already does at present. Moreover, worldwide, use efficiencies of land, water, nutrients and energy would greatly improve when yields would no longer be reduced

by late blight. A major effort to realize this is warranted. In the Netherlands such an effort is being made within the so-called DuRPh programme (see below).

Identification, Exposure and Containment of the Causal Organism

The mycelium of the oomycete *Phytophthora infestans* produces a haustorium which grows in the intercellular space of the plant and does not penetrate the cell cytoplasm as this remains protected by the cell membrane. The haustorium produces effectors, proteins that are either recognized or not recognized by the potato plant once they pass through the cell membrane.

Many effector genes of the oomycete are in-planta-induced (ipi), and one of them is the RXLR effector gene *ipiO* (Birch et al. 2006). This gene family consists of at least 16 variants which can be classified into three classes: I, II and III, of which the latter does not induce the so-called hypersensitivity reaction (HR) when co-infiltrated with *Rpi-blb1* in tobacco. This means that it is potentially pathogenic.

For the effector (protein) molecule to pass the cell plasma membrane it needs the presence of the RXLR-EER motif (and a signal peptide) present at the N-terminus of the effector protein. The signal peptide suffices for secretion (out of the hyphae), and the RXLR motif is involved in translocation inside the plant cell.

Avirulence factors (Avr) are proteins (effectors) that are recognized by the host plant. If a host plant is carrying the corresponding *R* gene it responds with a hypersensitivity reaction (HR) in a so-called "gene-for-gene" action. For instance: *Phytophthora* pathotypes may contain Avr3a and then fail to heavily infect potato varieties with the *R3a* gene. So avirulence (AVR) proteins are effectors detected by the plant.

There is a continuously ongoing "arms race" between the plant and the oomycete (Birch et al. 2006). The oomycete requires a large pool of effectors and a rapid evolution of the effector genes in order to be successful in breaking resistance in the host plant. The *R* genes of the host plant (the potato) are under strong selective pressure to recognize the products of the effector genes of the oomycete, in order to be able to trigger the hypersensitive response necessary to call the spread of the pathogen within the plant to a halt. Polymorphism of *Avr* genes is the result (Soanes and Talbot 2008).

Research in this field is extensive and producing exciting results. Recently, Jiang et al. (2008) showed that the RXLR effector reservoir in two species of the genus *Phytophthora* is dominated by a single rapidly evolving superfamily with more than 700 members. Sequencing the oomycete genome and searching for the N-terminal RXLR-EER motifs yielded some 700 effectors of *Phytophthora infestans*. They all belong to a single superfamily termed avirulence homolog (*Avh*) genes and show extensive sequence divergence and polymorphism, but yet they are all related and likely evolved from a common ancestor.

Scientists at Wageningen University (Wageningen, the Netherlands) and the Scottish Crop Research Institute (Invergowrie, UK) screened these 700 effector genes and found that some 400 did not produce RNA in planta and therefore they cannot produce effectors during infection by *P. infestans*. The other 300 effector genes, which do show expression in planta, are cloned in *E. coli* and then transferred to *A.*

tumefaciens, and infiltrated in tobacco simultaneously with a cloned *R* gene (co-infiltration, agro-infiltration, transient assay). When a hypersensitivity reaction occurs in the tobacco leaf the potato *R* gene recognizes the effector meaning that the interacting *Avr* gene is identified. A potato carrying that specific *R* gene is then resistant against this specific pathotype containing the *Avr* (Vleeshouwers et al. 2008).

When three genes are stacked, e.g. *Rpi-sto1* + *Rpi-blb3* + *R3a*, the *Avr* genes matching the three *R* genes are infiltrated one by one (separately) into the potato leaf. When the *R* gene recognizes the effector, an HR is shown. When three genes are stacked, a functional expression of the *R* genes can be detected simply by infiltrating the Avr and monitoring for an HR. Note the dose (concentration) of the effector in the transient assay is important as it is indicative of the level of resistance. The higher the dose needed to show an HR the earlier late blight will develop in the field. This method is now being further developed as a tool to predict *R*-gene based resistance.

When *Phytophthora* isolates are collected from different fields it is still necessary to carry out a detached leaf test on a differential set of potato genotypes with known presence of *R* genes rather than screen them for effector presence because ultimately, the detached leaf test is proving whether the present *R* genes confer resistance to *P. infestans*. Once the *R-Avr* interactions are verified by disease tests and agro-infiltrations, more high-throughput strategies on *Avr* monitoring based on DNA can be designed.

Principles of Durable Resistance Against *Phytophthora infestans* Using Potato Genes Only

The outbreak of late blight in Europe more than 150 years ago gave an impulse to potato breeding. In conventional breeding pollen of a high yielding potato variety is used to fertilize a variety which possesses a desired trait, for example resistance to blight. Over the last 100 years many times a breeder thought that late blight resistance was achieved, especially when a resistance gene of the wild species *Solanum demissum* was bred into the new variety. But when the new variety was grown for some years and at some scale, the resistance was always broken down. Compatible spores from elsewhere or from a mutation of the oomycete managed to infect the crop and in a few years sufficient inoculum (spores) was built up to successfully infect the crop soon after emergence. After 100 years of breeding no progress was made with the introduction of single genes from the wild potato species *Solanum demissum*.

Introducing genes from other wild species takes up to 50 years to result in a variety and then it is still hard to improve the old ones. Varieties Russet Burbank from the USA and Bintje from the Netherlands, both more than 100 years old, are still grown widely because of superior quality and because growers have the chemical means to control blight.

Introgression breeding of genes other than from *S. demissum* is an option to arrive at resistant varieties. In recent years, two resistant cultivars were released that presently find their way in organic potato production in the Netherlands. Figure 3 schematically shows the trajectory of 46 years from the first bridge crosses to the recently released varieties Bionica and Toluca.

There is, however, concern to continue to grow potatoes with its losses and costs associated with late blight as described in the previous section. The potential advantages of a late-blight free potato cultivar in terms of environment, human health,

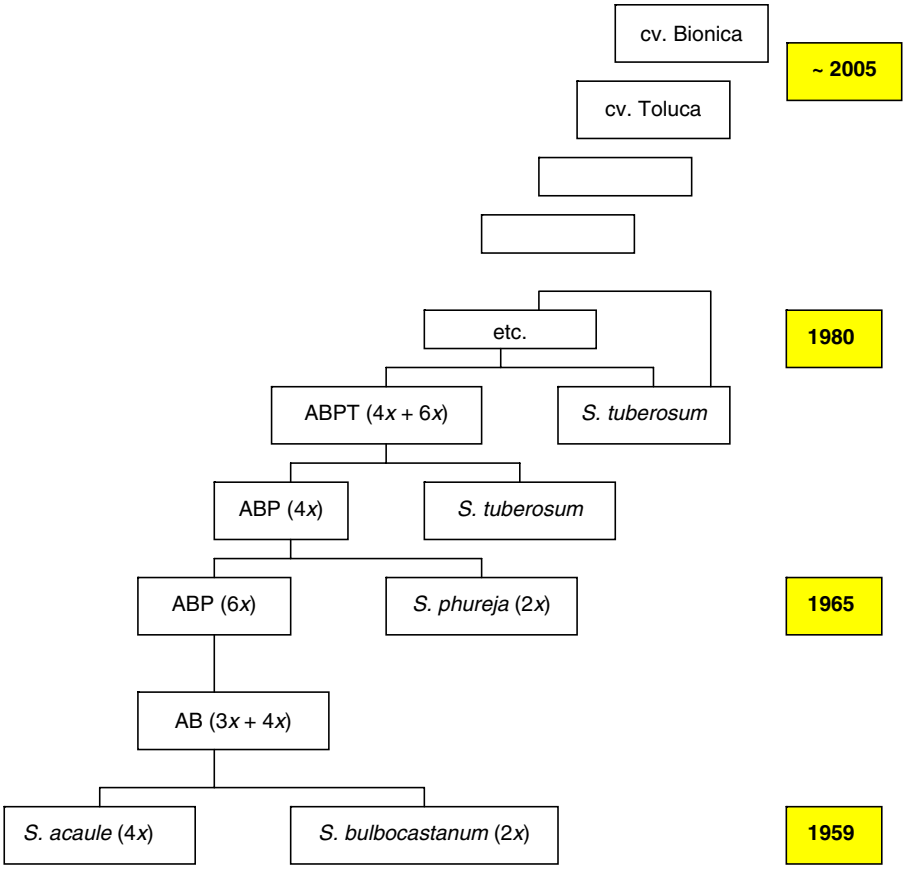

Fig. 3 Scheme of interspecific bridge cross breeding activities with late blight resistant *S. bulbocastanum* at Wageningen University and Research Centre and private breeding companies in the Netherlands. After 46 years the first resistant varieties Bionica and Toluca appeared, containing the single broad spectrum resistance gene *Rpi-blb2*. Note that stacking of *R* genes through this approach would even be more complicated and slow

food security and economic profit prompted the Netherlands government in 2006 to subsidize (with € 1 million/yr for 10 years) the efforts of Wageningen University and Research Centre to create a potato with **Du**rable **R**esistance against **Ph**ytophthora: the DuRPh project. The pronunciation of the acronym sounds as the Dutch word for courage. For a previous report on DuRPh see also Haverkort et al. (2008).

The DuRPh Programme

The DuRPh programme maintains eight basic principles to achieve durable resistance against the disease:

1. DuRPh applies **genetic modification** (GM) as a breeding technique whereby genes in wild species are detected, isolated and cloned and subsequently

transformed into existing varieties using the bacterial vector *Agrobacterium tumefaciens*. The resulting plantlets regenerated from callus are screened to assess whether they are 'true to type'. They should have the same phenotype as the—wildtype—variety into which the *R* genes were introduced. The resulting resistant potato varieties contain potato genes only and no bacterial genes. In theory one could achieve the same result by making crosses as is done in conventional breeding but it would take unacceptably long—especially to introgress several *R* genes and one would still not have exactly the same varieties as currently favoured by consumers and processors.

2. A **cisgene** approach. DuRPh only uses natural resistance genes from the plant itself or from crossable species from wild potato species that are crossable with cultured potatoes. Resistance could also be achieved by transgenic modification. The choice for cisgenic rather than transgenic was based on the availability of *R* genes in the potato pool and because it was assumed that this approach was ethically more acceptable to the public than the use of alien genes from not crossable species (cf. Jochemsen 2008), at least in conventional production. The organic movement is likely to maintain its principle objections against this method of breeding (Lammerts van Bueren et al. 2008), as cisgenesis does not comply with the principles of intrinsic value and the integrity of plants (Lammerts van Bueren et al. 2003).

   The researchers also favour the use of cisgenes in order to obtain exemption with the cisgenic plants from the GMO directive 2001/18/EC (Jacobsen and Schouten 2007). At this moment (July 2009) an EU committee is installed to evaluate technical considerations regarding new techniques such as cisgenesis with respect to application of directive 2001/18/EC.
3. To avoid what happened in the past when resistance of a single gene was broken rapidly, several resistance genes are inserted—up to four—of different wild species (**gene stacking** or **pyramiding**) to decrease the likelihood of rapid break-down.
4. DuRPh does not use selection markers such as herbicide tolerance or antibiotic resistance to select for insertion of the desired gene(s) in the variety. The achieved late blight resistance is the main marker so the variety is **marker free**. To assure that the *R* gene is (or the *R* genes are) present the genotypes that appear resistant to all known pathotypes of *Phytophthora infestans* are tested using PCR techniques.
5. It is well known that late blight caused by *Phytophthora infestans* in the past managed to bypass resistance. So to avoid that even stacked resistance genes lose their immense potential value, DuRPh intends to deploy them not in all varieties at all places at the same time but envisions a **resistance management** strategy whereby various combinations of stacked genes are deployed in different varieties at different sites at different times. It might well be that in future a mixture of a variety with different stacks of *R* genes is present in the field and that a set is used for say 6 years and then shelved for the following 6 years before being used again, preferably in a different region, thus resulting in spatially and temporally separated deployment of *R* genes.
6. Within the DuRPh strategy no new varieties are created but **the old varieties are kept** and are only provided with a cassette of resistance genes of wild potato

species. The start and end products are potato varieties made up of potato genes only.

7. DuRPh dedicates a considerable proportion of its efforts to **communication** with all stakeholders concerned. By being transparent regarding philosophy, approaches, techniques, and results it is up to individuals and groups to make their own decision about appreciation of the techniques used.
8. The **exploitation** of the *R* genes with proven efficacy and known not to be homologous to previously discovered ones is by protecting the intellectual property and making them available (not exclusively) to private potato breeding companies; once these genes are exploited in new varieties the private companies reimburse the owners through breeding right agreements. For developing countries where food security is an issue humanitarian aid through *R* genes is an option.

Approach and Work Packages of the DuRPh Programme

The basic aim of the DuRPh programme is to deliver the proof of principle that a cisgenic potato variety can be made which is durably resistant against late blight. The research and development needed to achieve that basic aim costs about one million Euros per year for 10 years. DuRPh has organized the R&D work in five subsidiary projects: cloning, transformation, selection, resistance management and communication. Below we describe what is done per sub-project (or work package) and how the different activities interact.

Cloning In this activity, researchers select for resistant species and make a cross between a susceptible and a resistant plant from the selected wild species. If the progeny segregates into two distinct groups of susceptible and resistant clones then it is apparent that there must be one (qualitative) resistance gene (*R* gene) responsible for the resistance reaction. For gene cloning, the approaches of map-based cloning and allele mining and all other possible approaches in between are used (Jacobsen and van der Vossen 2009). Figure 4 shows some of the candidate genes already cloned.

Fig. 4 Some of the candidate genes conferring resistance to *Phytophthora infestans* (*Rpi*) cloned at Wageningen University

Gene cloning

Map-based / candidate-gene cloning approaches

Species	Resistance gene
Solanum demissum	*R2, R3a, R3b*
Solanum bulbocastanum	*Rpi-blb1, Rpi-blb2, Rpi-blb3*
Solanum venturii	*Rpi-vnt1*
Solanum papita	*Rpi-pta1*
Solanum stoloniferum	*Rpi-sto1*
Solanum ?	*Rpi-abpt, R2-like*

Transformation In this activity the many identical clones of the *R* gene are transferred to another bacterium—*A. tumefaciens*—normally occurring in soils and known to infect trees and to genetically modify them triggering the formation of galls or tumors in which the bacterium thrives. This natural ability of the bacterium is used to transfer the cloned *R* gene (or a cassette of several *R* genes) to individual cells of leaves of a potato variety in a liquid suspension to induce transformation. The individual cells are allowed to multiply into groups of cells (callus) that under the influence of gravity form downwards roots and upwards a shoot. The regenerated plants are tested for late blight resistance and for the presence of the genes in the cassette. When three genes are stacked (e.g. *Rpi-xx1* + *Rpi-yy2* and *Rpi-zz3*) the *Avr* genes matching the three *R* genes are infiltrated one by one (separately) in the potato leaf. When the *R* gene recognizes the effector, a hypersensitive response (HR) occurs. When three genes are stacked, a functional expression of the *R* genes can be detected simply by infiltrating the Avr and monitoring for an HR (Vleeshouwers et al. 2008). Figure 5 shows the single and multiple resistance genes used with and without a selection marker. The selection marker containing plants are only used to quickly assess the best possible combination of *R* genes after which these genes are introduced into the desired potato variety by a marker free approach as described by de Vetten et al. (2003). *P. infestans* resistant plants move to the next sub-project.

Selection In this activity, the plants that are generated in the way described above may or may not contain the desired gene(s). Moreover, although often genetically identical, they may (slightly) differ from the original variety (wild type) because they

Durable resistance against *Phytophthora infestans*

R-gene cassettes

Made constructs or construct combinations (*with selection marker*)

- *Rpi-blb1*
- *Rpi-blb2*
- *Rpi-blb3*
- *R3a*
- *Rpi-sto1*
- *Rpi-blb1* + *Rpi-blb2*
- *Rpi-blb1* + *R3a*
- *Rpi-sto1* + *R3a*
- *Rpi-sto1* + *Rpi-blb3*

Made constructs or construct combinations (*marker free*)

- *R3a*
- *Rpi-sto1* + *Rp1-blb3* + *R3a*

Fig. 5 Examples of single and stacked resistance genes used within the DuRPh project to date (mid 2009)

went through a callus phase leading to so called somaclonal variation. Within the sub-project 'selection' researchers look for the genotypes that contain the desired resistance gene(s) of the gene cassette. The plantlets or their leaves are subjected to known races of late blight and the resistant ones are also subjected to a molecular technique (PCR) to make sure that the sequenced gene is actually present. The other way around is first PCR-selection followed by resistance testing. This way no marker gene with e.g., antibiotic (such as kanamycine) resistance is needed to prove the presence of the desired gene. Individual plantlets of the new resistant genotype are then allowed to grow into a plant with tubers and it is made sure that the selected ones have the same or similar characteristics (e.g., flowering time and flower colour, shape, cooking and frying quality) as the original variety.

Resistance Management DuRPh wants the new resistant variety to have all the properties of the original one based on the concept of a 'dynamic variety'. This concept means that at different places and times the variety contains cassettes of *R* genes of varying composition. Using past experiences of resistance break-down, sampling of various late blight races in different regions of the Netherlands and Europe and the expected effect of pyramiding, and with the aid of computer simulations of scenarios of population dynamics, this sub-project comes up with schemes of cassettes of stacked *R* genes deployed in different varieties, times and spaces. Potentially in future some genes are used for some time in an area, then moved to another variety in another area or withdrawn altogether for some years before being reintroduced again. The reserve of *R* genes is not endless so they have to be kept intact and effective as long as possible.

Communication In this activity, DuRPh scientists explain to 'whomever it concerns' what they do and how they do it. The stakeholders informed are scientists, non governmental organizations, the farming community, potato breeding companies and the seed potato industry and consumers. DuRPh wants all stakeholders to be able to inform themselves and to provide them with all the information needed for them to make sound, informed decisions. It may influence the public's resistance against genetic modification—at least against some forms of this type of biotechnology. This sub-project also informs decision makers, e.g., in the European Union in Brussels, to rethink legislation around genetically modified crops that was based upon the modification of plants whereby genes from bacteria, viruses, other non-crossable plant species or synthetic genes were introduced into plants. All such transgenic plants until now had alien selection genes coding for antibiotic resistance or herbicide tolerance. Legislators are invited to consider exempting cisgenic marker-free potato from the scrutiny of transgenic plants. Exemption from heavy scrutiny and lengthy and costly testing that relatively favours large international companies has taken place before: induced mutation breeding with the aid of chemicals or atomic radiation is considered genetic modification by the EU but is exempted and so is protoplast fusion between crossable species (Jacobsen and Schouten 2007). If cisgenic marker-free breeding were treated similarly or with a light procedure also small and medium sized companies such as the Dutch potato breeding cooperatives could afford introducing genetically modified plants.

Fig. 6 "Première" provided with an *R* gene (with leaves) besides its wild type with leaves decayed because of late blight infection (3 weeks after inoculation in July 2008). Photograph: A.J. Haverkort

Outlook

The present R&D project costs about one million euro per year. If proven successful and when legislation becomes favourable regarding cisgenesis, commercial breeders could do the same work for a fraction of this amount and relatively quickly 'upgrade' their varieties, thereby increasing profits and reducing the strain on the environment. Figure 6 exemplifies the impact of genetic modification. The trial in 2008 contained a single *R* gene and still contained a selection marker. The 2009 field trial for the first time had two *R* genes stacked and contained the first marker-free event.

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