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Initiatives on Potato Functional Genetics

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ABSTRACT

Potato is the third most important food crop worldwide. The identification of genes or chromosome regions responsible for agronomic traits had been extensively attempt in the past 20 years, mainly for disease resistance and tuber traits. Despite the success of these studies, only few discoveries were incorporated to plant breeding programs or used in surveying germplasm. With the forthcoming complete genome sequence of potato we envisaged that gene identification and *in vivo* functionality will be dramatically increased, given the availability of genetic sequences of potato and tools to assess gene function. Assisted breeding based on markers derived from these genes will be in an ideal situation where cross-over cannot occur between the marker and the gene responsible for the trait. Moreover, genetic diversity of germplasm banks can be assessed in terms of allelic variability of important genes. In this review, we present some of the studies carried out for potato functional genetics, and our contribution in the development of tools for transient silencing. The lessons learned from functional studies can also be applied to other *Solanaceae* such as tomato, eggplant, petunia, tobacco and pepper, which present a high level of synteny with potato.

Keywords: functional map, potato genome, VIGS, P450, P69
Abbreviations: **AFLP**, amplified fragment length polymorphism; **DM**, doubled monoploid; **GTMs**, gene targeted markers; **PGSC**, Potato Genome Sequencing Consortium; **PTGS**, post-transcriptional gene silencing; **PVX**, *Potato virus X*; **PVP-Ar**, potato virus P strain Argentina; **PRDV**, *Potato rough dwarf virus*; **QTLs**, quantitative trait loci; **RFLP**, restriction fragment length polymorphism; **SNP**, single nucleotide polymorphism; **SSR**, single sequence repeat; **TRV**, *Tobacco rattle virus*; **UHD**, ultra high density; **VIGS**, virus-induced gene silencing

CONTENTS

INTRODUCTION

Potato has become the third most important food crop, surpassing maize, based on the increasing production and consumption driven by the emerging economies of China and India (FAO 2010). It is estimated that by year 2020, more than two billion people in Asia, Africa and Latin America, will depend on this crop for food, feed and subsistence (Kuang *et al*. 2005). Moreover, potato synthesizes starch with unique properties for non-food applications, including biodegradable plastic substitutes (Avula and Singh 2009).

Potato is a member of the *Solanaceae*, an extensive family of more than 3000 species that besides potato includes very important crops as tomato, pepper, tobacco, eggplant and a large number of wild relatives that constitute a reservoir of novel genes and alleles.

Potato is one of the most genetically diverse cultivated plants. In the Andean region of South America it is an ancient traditional crop (archeological evidence dates its cultivation to 7000 years ago (Engel 1984). Systematics of potato is still under debate and two main classifications had been proposed. One states that potato is represented by seven species (*Solanum tuberosum*, *Solanum stenotonum*, *Solanum ajanhuiri*, *Solanum chaucha*, *Solanum phureja*, *Solanum curtilobum* and *Solanum juzepczukii*) where *S. tuberosum* is subdivided in two subspecies: *andigena* and *tuberosum* (Hawkes 1994). A more recent approach (Huaman and Spooner 2002) postulates that potato is one species (*S. tuberosum*) comprising eight groups (Chilotanum Group, Andigenum Group, Stenotonum Group, Ajanhuiri Group, Chaucha Group, Phureja Group, Curtilobum Group, and Juzepcukii Group). The fact that gene flow between genotypes of these groups is possible supports the latter hypo-

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thesis.

All the above-mentioned species, including their wild relatives and hybrids of cultivated and wild plants represent a pool of genes for valuable traits, such as resistance to pests and diseases, adaptation to abiotic stresses and nutritional and industrial attributes. The existence of synteny between these species along with the increasing importance of potato as a relevant component of the human food and nourishment, actual and future, emphasize the importance of structural and functional genetic studies within this species and their close relatives.

The identification of genes responsible of key attributes of agronomic, nutritive, industrial and pharmaceutical importance, which will be soon reached by the sequencing of the potato genome and the understanding of their functions, will greatly enhance the potential of improving potato varieties by conventional breeding, marker assisted strategies and/or genetic modification approaches.

THE POTATO GENOME SEQUENCING CONSORTIUM

The potato genome comprises 12 chromosomes at the haploid level; with an average length of 840 Mb. Chloroplast and mitochondrial genomes add 150 and 450 Kb, respectively.

A large international effort for the complete sequencing of the potato genome has been set up with the collaboration of 13 countries from diverse regions of the world (China, India, Poland, Russia, the Netherlands, Ireland, Argentina, Brazil, Chile, Peru, USA, New Zealand and the UK). This initiative called the Potato Genome Sequencing Consortium (PGSC; http://www.potatogenome.net) was originally based on a traditional bacterial artificial chromosome (BAC) by BAC approach, supported by a recombination map of 3500 fragment length polymorphism (AFLP) markers from ultra high density map (UHD) derived from the diploid *S. tuberosum* SH83-92-488 x RH89-039-16 cross (SH × RH) (van Os *et al*. 2006). The genomic library (Imagenes, Germany) is based on RH and contains 78336 BAC clones of approximately 100-120 Kb each, making a 10X coverage of the estimated potato genome. All clones are fingerprinted by AFLPs and their end sequences are already publicly available in the genomic survey sequence database of NCBI (http://www.ncbi.nlm.nih.gov). AFLP bands were applied to assemble almost 40% of the BACs in 7000 contigs, with around 1600 of them with known position on the UHD map (Visser *et al*. 2009). Both, AFLP fingerprints and end sequences are utilized to define a map of contiguous overlapping BAC clones, called contigs, with the aid of the program FPC (Soderlund *et al.* 2000); and set the BAC minimum tiling path (Visser *et al*. 2009), which is estimated to comprise 10,000 BAC clones with an overlap between BAC clones of 10-20% (Visser *et al*. 2009).

The association of BACs to chromosomes allowed the distribution of the workload between the partners of PGSC based on chromosomes. Chromosome III was assigned to the PGSC South-American group formed as a strategic alliance between Peru, Brazil, Chile and Argentina, with the additional objective of enhancing training capabilities in genomics and bioinformatics in the Region.

Chromosome III marker density in the UHD map was low including only 124 of the 3413 of the RH map (van Os *et al*. 2006). Only 33 of the 80 bins defined by cross over events contained AFLP markers (Visser *et al*. 2009). Consequently, the number of BACs contigs assigned to chromosome III was less than 60, consistently lower than other chromosomes. Out of these, at time of writing, 18 seed BACs (BACs that represent a contig) have been sequenced by the South American group.

Despite the skewed distribution of markers on chromosomes, dissimilar advance had been obtained for different chromosomes, by different partners due to several factors such as date of inception, budget, bioinformatics and script development capacities and familiarity with tailor developed tools and data. Moreover, the heterozygous nature of RH brought additional inconveniences especially on the assembly of low complexity orthologous regions. The advent of new parallel sequencing technologies led to a significant change of approach within the PGSC and in 2008, the consortium initiated sequencing of the doubled monoploid DM1-3 516R44 (DM) potato derived from a diploid landrace of potato (*S. phureja*) in order to simplify and complement the RH effort. This new approach, based on shotgun sequencing of DM, made the first draft of the potato genome publicly available by the PGSC at http://www.potatogenome.net at the time of writing this review. Subsequent updates will include annotation of the genes, analysis of when and where they are switched on and off. The outcome of this international effort will produce a high number of candidate genes that are critical to potato production, however, their *in vivo* function cannot be directly inferred from homology with previously identified genes, and will require additional approaches with the aid of special tools and experiments.

FUNCTIONAL MARKERS AND MAPS

Despite its importance for human food and nutrition, very little is known of genes responsible for important traits in potato. The genetic structure of this crop (tetraploid, highly heterozygous, self-incompatible) hampers the use of inbred isogenic lines, single mutant collections and other tools to help in gene function determination.

Molecular DNA-based markers were originally developed from non-genetic regions to exploit the highly polymorphic sequences stochastically accumulated by mutations not subjected to selection present in these areas.

There was an extensive development of linkage maps and location of single genes and quantitative trait loci (QTLs) using these non-genic DNA markers such as restriction fragment length polymorphism (RFLPs), AFLPs and SSRs, rendering putative regions linked to disease resistance (for a review see Gebhardt and Valkonen 2001); tuber shape (van Eck *et al*. 1994a) tuber skin color (van Eck *et al*. 1993, 1994b) tuber starch content (Bonierbale *et al*.1993; Douches and Freyre 1994; Schäfer-Pregl *et al*. 1998) and cold sweetening (Menéndez *et al*. 2002) among other agronomic important traits. In very few cases, the responsible gene or genes underlying the QTL (or major locus) effect was identified and characterized.

Functional molecular markers, or markers developed from genetic sequences, can be envisaged as a first approximation of functional genetics, as candidate gene approaches can be attempted from co-localized genetic markers with QTLs or from knowledge of biochemical pathways involved in key traits.

These markers can be derived from single nucleotide polymorphisms (SNPs) identified in genes (or gene families) or by the occurrence of suitable DNA motifs, as microsatellites or restriction sites, to design successful markers.

Andersen and Lübberstedt (2003) defined different categories for functional markers discriminating between gene targeted markers (GTMs), indirect functional markers (IFMs) and direct functional markers (DFMs). Under this classification, markers designed from genes bearing SSR motifs would be considered as GTMs, and markers from candidate genes causally linked to phenotypic traits would be either DFMs or IFMs, depending whether the assessment of functionality of the gene/allele is confirmed or not.

Genetic functional maps can be assembled by the use of sufficient number of functional markers. These maps would aid in the study of complex characters with multiple QTLs where the relationships between them can be established under a biochemical and/or physiological view of the enzymes coded by the functional markers, setting up a framework for metabolic engineering of plants. Marker assisted breeding based on DFMs or IFMs would count with ideal markers, where crossover between the marker and the gene responsible of the desired trait will never occur, as they are

the same. Moreover, genetic resources of Germplasm Banks can be weighted based on the intra- and inter-specific allelic diversity for key genes.

GENIC SSRs

The increasing amount of cDNA sequences in public databases in the last few years, and the availability of bioinformatic tools to search for certain motifs, stimulated the relatively inexpensive development of EST based SSRs in different crops (Varshney *et al*. 2005).

In potato, 61 EST-SSRs were developed mining the former TIGR potato EST database (now "The Gene Index Project"; http://compbio.dfci.harvard.edu/tgi/), and 56 of them were located in a joined *S. berthaultii x S. tuberosum* diploid framework map (**Fig. 1**; Feingold *et al*. 2005). The markers developed and characterized in this study, do not only provide reproducible co-dominant, easily scored markers, but also may include candidate genes that have the potential of being causally linked to the trait of interest. Accordingly, these GTMs can be directly included in functional maps and based on the putative function of the clones they have been generated from, may be helpful to understand a causal-effect relationship in co-located, previously mapped QTLs. For example, StI002, designed on a putative invertase gene, maps to chromosome 9 in the same region of a previously identified QTL for tuber sugar content (Menéndez *et al*. 2002). After these evidences, Colman *et al*. (2009) evaluated the allelic diversity of 25 different genotypes of both *S. tuberosum ssp. tuberosum and ssp. andigena* from the INTA Balcarce Germplasm Bank. Out of the nine invertase alleles found, two were associated with chip quality. StI002_9 was associated with bad chip quality (high reducing sugar content) in the Tuberosum genotypes and StI002_6 was associated with better chip quality in the Andigena genotypes, the last one being exclusive of Tuberosum genotypes (**Fig. 2**).

Another advantage of the EST-SSRs is their transferability across species (Scott *et al*. 2000). In this respect, eight of the SSRs developed in this study were chosen by the International Potato Center (CIP-CGIAR) as part of a "Genetic Identity Kit" of 24 markers for fingerprinting studies in diverse potato germplasm (Ghislain *et al*. 2009). Some of these markers were also useful in assessing genetic variability within and between entries of 'Collareja' (*S. tuberosum ssp. andigena*) a traditional variety of NE of Argentina (Atencio *et al*. 2008, 2009).

FUNCTIONAL MAPS

Chen *et al*. (2001) developed cleaved amplified polymorphic site (CAPS), sequence characterized amplified region (SCAR) or RFLP markers from 69 genes related to the carbohydrate metabolism and transport, that defined 85 genetic loci in an integrated map of four diploid populations. The resulting functional map was compared with previous QTL maps for tuber starch content (Schäfer-Pregl *et al*. 1998) and only three of the positions of the 17 QTLs did not match any candidate-gene loci. These results validate the functional marker-QTL co-localization strategy to identify candidate genes and as a first step in functional assessment.

Cytochrome P450 monooxygenases (P450s) is the largest and probably the most diverse gene family of plants. While in *Arabidopsis thaliana* 272 genes (including pseudogenes) have been identified, potato P450 superfamily was estimated to comprise as high as 400 genes. P450 enzymes catalyze oxidation on different substrates and have been shown to be involved in the biosynthesis of alkaloids, anthocyanins, fatty acids, flavonoids, gibberellins, polyphenolic acids, steroids and terpenes. Some of the *in vivo* function of these metabolites is related to detoxicative processes against herbicides, UV light protection and insect defense (Szafranek and Szafranek 2008). Thus, the potential for discovery of useful genes in this family is high. Many P450 genes have been isolated via PCR amplification using primers to conserved regions, or random cDNA sequencing. We have searched theformer TIGR potato and tomato EST databases (http://compbio.dfci.harvard.edu/tgi/) using 11 *Arabidopsis thaliana* P450 sequences that represent most of the plant P450 clades. 486 tentative consensus and singleton sequences (238 from potato and 248 from tomato) retrieved at time of producing this study, were identified. As many of them may represent orthologs between the two species, closely-related paralogs or allelic sequences, putatively unique sequences were identified by alignments and primers were designed for 125 "unique" P450 genes. PCR products revealing single nucleotide polymorphisms (SNPs) between parental genotypes of two diploid breeding populations have been used to map P450s using SSCP, allele-specific assays, or direct sequencing. One hundred and six putative non redundant P450 genes have been mapped to all of the 12 potato chromosomes (**Fig. 1**) in BCB and BCT frame maps (Bonierbale *et al*. 1994; Feingold *et al*. 2005). Some of these genes were found in clusters in many of the chromosomes (**Fig. 1**). The chromosome location of members of the same family can also be informative in gene evolution studies, as there is a lot of evidence that genes may evolve through tandem duplication (Jander and Barth 2007). However, we are aware that some of the markers may correspond to different regions of the same gene, since PCR primers were designed based on available sequence, sometimes partial, at the time of the study. The recent release of the complete potato genome can be used to identify such events.

Original tentative consensus and singleton identifiers, amplification primers and source sequences of the mapped P450 genes, are available as AEM (http://www.inta.gov.ar/balcarce/Agrobiotecnología/Bases.htm). Some of these results were presented elsewhere (Feingold *et al*. 2004, 2005) but this is the first complete publication of these data.

Leptines are rare glycoalkaloids synthesized by few accessions of *S. chacoense* (Sagredo *et al*. 2006) and have long been associated to Colorado Potato Beetle (CBP; for a review see: Alyokhin 2009) resistance (Khun and Löw 1961; Sinden *et al*. 1986). Synthesis of leptines requires hydroxylation of solanidine (the normal aglycone of *S. tuberosum* glycoalkaloids) in C-23 followed by the acetylation of the resulting hydroxyl group (Sagredo *et al*. 2006). A QTL for leptidine synthesis was mapped in a tetraploid population in a distal location of chromosome 2 (Sagredo *et al*. 2006). These authors postulate that a P450 monooxygenase would be a likely candidate gene for a structural gene, and based on **Fig. 1**, any of the three P450 markers located on top of chrosome II could be further studied to test this hypothesis.

Subtilisin-like serine peptidases, also called subtilisins, are a subfamily of the MEROPS family of Serine proteases (S8A, http://www.merops.sanger.ac.uk), which have been related to biotic plant defense in plant, development, tissue differentiation and senescence (for review see Antão and Malcata 2005).

Jordá *et al*. (1999) isolated and characterized four tomato p69 genes distributed in two clusters in a tandem array, a cluster with P69A to P69D; MEROPS S08.006) and other cluster with P69 E and F (Jordá *et al*. 2000). At the same time, Meichtry *et al.* (1998) described 15 subtilisins from tomato sequences called LeSBT1, LeSBT2, LeSBT3, LeSBT4A-D, TMP and 6 P69 (P69A-F). Among these, P69B and P69C has been related to defense, both are differentially expressed under *Pseudomonas syringae* infections and up regulated under salicylic acid molecules that trigger mechanisms. (Jordá *et al.*1999). P69B is also induced in response to citrus exocortis viroid (Zhao *et al*. 2003; Tornero *et al.* 1997) and is specifically recognized and inhibited by two extracellular elicitors from *Phytophthora infestans*, EPI 1 and EPI 10 (Tian *et al.* 2004, 2005). Interestingly, more than the half of Pi inhibitors (including EPI 1 and EPI 10) present the cleavage motif of P69 subtilisins (Tian *et al*. 2004), suggesting that more proteases could be involved in *P. infestans* defense.

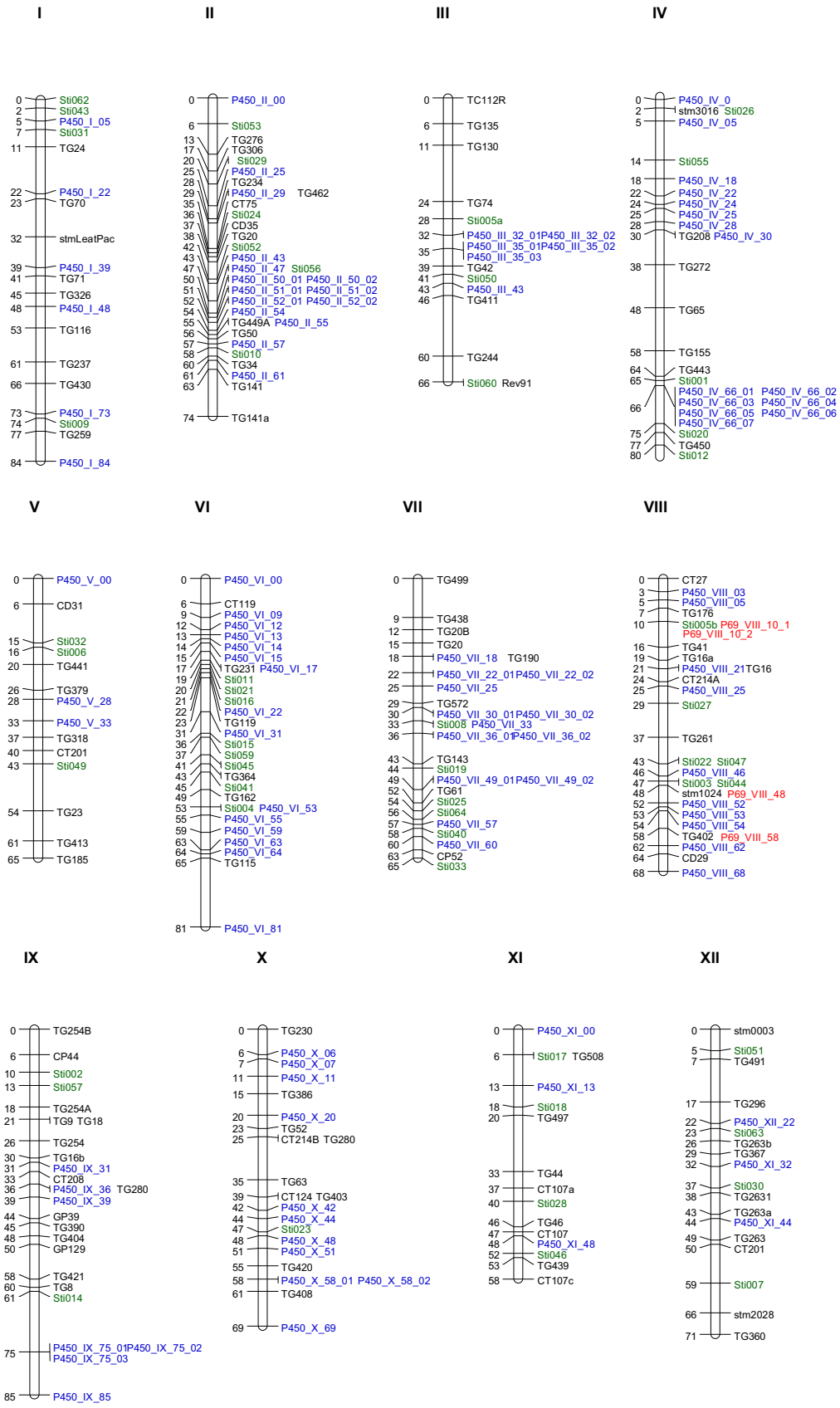

Fig. 1 Location of 55 mapped expressed sequence tag derived simple sequence repeats (EST-SSRs) and 106 putative non-redundant P450 genes derived markers, in a joined restriction fragment length polymorphism potato genetic map derived from BCB and BCT populations (Bonierbale *et al.* 1994). EST-SSRs are in green and indicated by StI code. P450s markers are in blue and indicated by the P450 prefix, followed by the chromosome location. The map position of these markers is indicative at the level of chromosome arm. Additional data for primers and source sequences is available on: http://www.inta.gov.ar/balcarce/agrobiotecnologia/Bases.htm

In order to identify potato P69 genes, we have used tomato p69 subtilisin-like gene sequences as queries to identify homologues in potato cDNA databases (DFCI, http://compbio.dfci.harvard.edu/tgi/ former TIGR database, Release 11; Genbank http://www.ncbi.nlm.nih.gov and Sol genomics network, http://www.sgn.cornell.edu/tools/blast/).

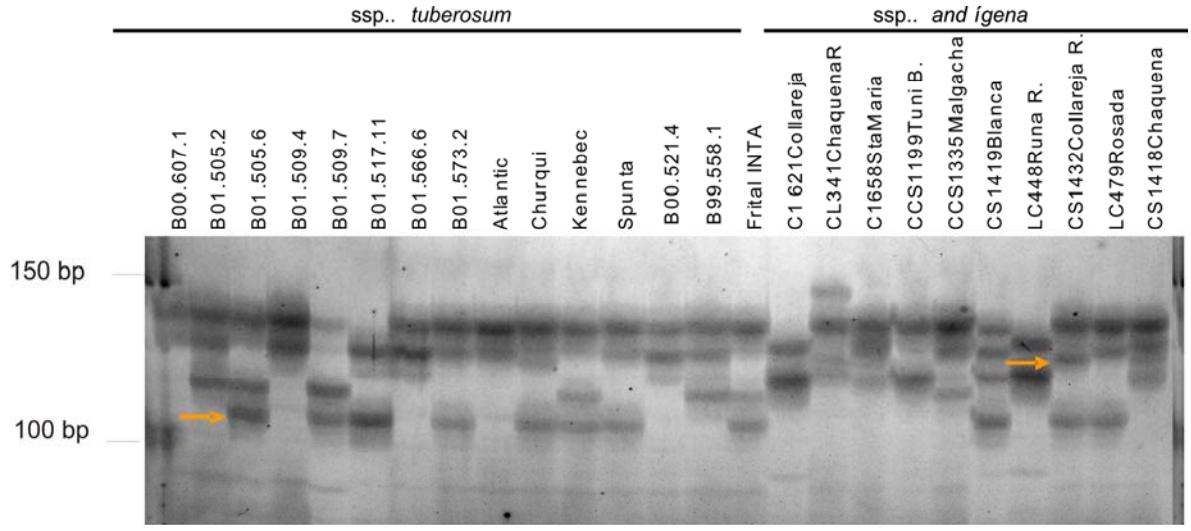

Fig. 2 Allelic variation of microsatellite StI002 designed on a putative invertase gene of chromosome 9 (from Feingold *et al.* 2005) across 25 *Solanum tuberosum* genotypes. Orange arrows indicate alleles associated with good (right arrow) and bad (left arrow) chip quality. Adapted from Colman *et al*. (2009).

Twelve putative non redundant potato P69 sequences (which can be access to http://www.inta.gov.ar/balcarce/agrobiotecnologia/Bases.htm) were retrieved. Four of them were located in chromosome 8 (**Fig. 1**), but unlike tomato, where P69s are completely linked (GenBank: AP009276.1, clone C08HBa0165B06), two pairs of P69 sequences were linked at distal ends of chromosome 8 (unpublished data; **Fig. 1**). Interestingly, several independent studies have placed QTLs for *P. infestans* on potato chromosome 8 near RFLP markers TG41, TG261 and microsatellite stm1024 (Ewing *et al*. 2000; Naess *et al*. 2000; Ghislain *et al*. 2001; Sorensen *et al*. 2006) in populations derived from germplasm as diverse as *S. tuberosum*, *S. berthaultii*, *S. vernei* and *S. bulbocastanum*. All these evidences suggest that P69s can be involved in the potato- *P. infestans* interaction. This hypothesis is currently being tested in our lab by expression and silencing experiments in susceptible and tolerant challenged genotypes.

FUNCTIONAL GENETICS AND GENE SILENCING

Functional genomics emerges as consequence of the vast amount of genetic information produced by sequencing projects with the objective to establish the function of newly discovered genes. Reverse genetics investigates the function of a gene or DNA sequence by directly altering the expression of the gene of interest and then identifying the mutant phenotype that is produced. In this sense, there are several approximations to assess gene function as complementation and/or over expression transgenic assays, gene expression chips, point mutant collections, but many reverse genetics approaches described in plants to date, rely on post-transcriptional gene silencing (PTGS) (Bouché and Bouchez 2001; Bradley *et al*. 2004; Xu *et al*. 2008).

Gene silencing is a homology-dependent process that causes the reduced (partial or total) expression of a gene, and is classified into two classes: Transcriptional gene silencing (TGS) and PTGS. TGS occurs at the DNA level and is characterized by changes in chromatin structure that reduce or prevent transcription. TGS could be the result of methyllation or changes in chromatin associated proteins. PTGS is a process that reduces cytoplasmic RNA levels whereas the transcription takes place but the mRNA is not accumulated in the cytosol preventing its participation in the protein biosynthesis (Meyer and Saedler 1996; Matzke and Matzke 2000).

VIRUS-INDUCED GENE SILENCING

Virus induced gene silencing (VIGS) is a technique that involves recombinant viruses for suppression of gene expression (Baulcombe 1995; Ruiz *et al*. 1998). Kumagai *et al*. (1995) reported the first PTGS mediated by recombinant tobacco mosaic virus (TMV) in plants. From these pioneering studies, VIGS technology was rapidly recognized as a tool for functional genetics, due to the possibility of silencing transcripts of specific genes and the assessment of function through phenotype testing (Baulcombe 1999). Moreover, due to the transient nature of the test, a great quantity of genes can be assayed, without the need of stable genetic transformation events. Advantages of VIGS include: i) identification of a loss-of-function phenotype for a specific gene within a single generation, ii) possibility to compare the gene function between different species, iii) low implementation costs and iv) needless generation of transgenic plants (Burch-Smith *et al*. 2004; Godge *et al*. 2008). Other important feature of VIGS is the easiness of vector introduction in plants mediated by *Agrobacterium tumefaciens* through a binary plasmid system with a modified T-DNA carrying the target sequence (Ratcliff *et al*. 2001; Liu *et al.* 2002). For these reasons VIGS is the preferential choice for functional genomics studies at large-scale.

Many VIGS vectors have been developed over the past 10 years; these vectors are based on different viral genus, from *Hordeivirus* that infect monocots to *Potexvirus* and *Tobravirus* that infect a wide range of solanaceous species (Godge *et al*. 2008).

There are two VIGS–based vectors that have been reported for potato gene silencing: *Potato virus X* (PVX, *Potexvirus*) (Ruiz *et al.* 1998) and *Tobacco rattle virus* (TRV, *Tobravirus*) (Ratcliff *et al.* 2001; Liu *et al*. 2002).

PVX-based vector, however successful in some studies (Faivre-Rampant *et al.* 2004), presents some limitations such as: i) limited host range (Brunt *et al*. 1996), ii) leaf symptoms that can mask silenced phenotypes (Ratcliff *et al*. 2001) and iii) silencing can be lost through the exclusion of the vector from meristematic tissues (Hull 2002; Ratcliff *et al*. 2001).

The TRV-based vector is widely used on *Solanaceae* because it presents many advantages over PVX as i) a wide host range ii) uniformity in the silencing, iii) reaches meristematic areas and iv) induces mild or none virus symptoms (Ratcliff *et al*. 2001; Liu *et al.* 2002).

Based on these antecedents, our group started working with TRV-based VIGS vectors. Two TRV vector systems were compared, one kindly provided by Dr. Baulcombe (Sainsbury Laboratory JIC, UK; Ratcliff *et al*. 2001), and another gently provided by Dr. Dinesh Kumar (Yale University, USA; Liu *et al*. 2002). These systems were identified for simplicity as in Brigneti *et al.* (2004) as System "A" and

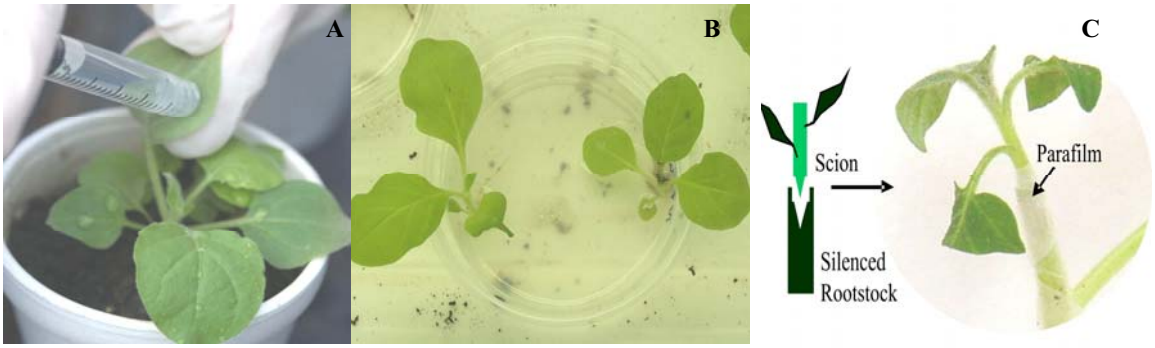

Fig. 3 Deliver systems used on TRV-based VIGS vectors carried on *Agrobacterium tumefaciens* (GV3101). Each picture represents the technique used with different plant material. (**A**) Agroinfiltration of 6-weeks-old *N. tabacum* (cv. 'Samsung') plant; (**B**) Agroimmersion of 4 weeks old *N. tabacum* (cv. 'Samsung') plant; (**C**) grafts between a 2 weeks old potato plant (cv. 'Pampeana INTA') scion, considerer from tuber planting, and a 5 weeks old *N. tabacum* (cv. 'Samsung') 3 days TRV (empty or with insert) inoculated rootstock.

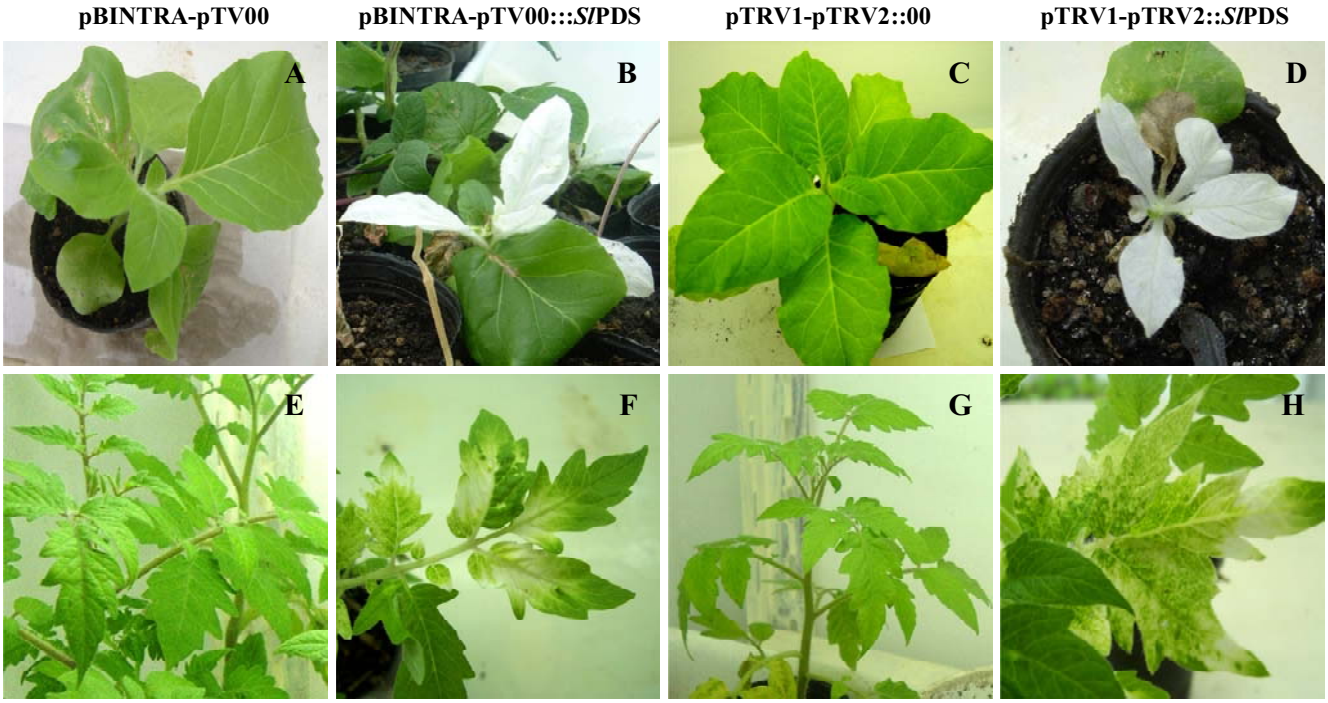

Fig. 4 PDS gene silencing on tobacco and tomato. Tobacco (**A, B, C, D**) and tomato (**E, F, G, H**) plants were agroinfiltrated with *A. tumefaciens* (strain GV3101) carrying TRV-based VIGS vectors: pBINTRA - pTV00::00: TRV system "A" without inserts, pBINTRA - pTV00::*Sl*PDS: TRV system "A" with PDS tomato insert, pTRV1-pTRV2::00 TRV system "B" without insert, pTRV1-pTRV2::*Sl*PDS: TRV system "B" with PDS tomato insert. Pictures **A, B, C, D** were taken 42 days post inoculation (dpi), while **E, F, G, H** 35 dpi.

System "B", respectively. Both systems are very similar, being the main difference the presence of a double 35S promoter in the RNA2 and a ribozyme in 3´end of the non – translated sequence that allegedly improves processing of the mature transcript on system "B" (Liu *et al*. 2002). *Nicotiana tabacum* and *Solanum lycopersicum* phytoene desaturase genes (*Sl*PDS and *Nt*PDS), were used as reporter genes. These homologous sequences share a high identity, with potato PDS partial cDNA sequence of 96 and 90%, respectively. PDS is essential for the production of carotenoids involved in UV protection (Demmig-Adams and Adams 1992), and the loss of function of PDS generates white tissue in presence of light, known as photobleaching (Kumagai *et al*. 1995). To test VIGS on cultivated potato, we used four tetraploid potato cultivars ($2n = 4x = 48$) (*S. tuberosum* ssp. *tuberosum* cvs. 'Pampeana INTA', 'Bintje', 'Atlantic' and 'Spunta' and 10 individuals from a *S. chacoense* diploid population ($2n = 2x = 24$) derived from BGRC-41479/1 × BGRC-41479/15 parents (all plant material was provided by the Germoplasm Bank from the EEA – Balcarce, Argentina). *N. tabacum* (cv. 'Samsung') and *S. lycopersicum* (cv. 'Platense') were used as positive controls. Three different vector introduction methods were assayed: i) agroinfiltration, ii) agroimmersion and iii) grafting (**Fig. 3**). Several parameters that could affect VIGS were initially assayed with agroinfiltration method: four levels of inoculum concentration (OD_{600}: 0.3; 0.6; 1 and 2), five plant phenological stages (2-, 3-, 4-, 5- and 6-week-old seedlings tobacco and tomato plants, and tuber-grown potato plants considered from tuber planting), two different *A. tumefaciens* strains (GV3101 vs. GV2206 strain) and the two mentioned TRV systems (System A and B). All plants were maintained at a temperature range from 18–20°C with a 16-h photoperiod. Both systems generated photobleaching in tobacco and tomato under most of the assayed conditions, but symptoms were not evident in any of the potato genotypes (**Fig. 4**). Photobleaching was first observed in growing leaves of tobacco and tomato control plants and was still evident even at 70 days post inoculation, when plants were discarded. Tobacco silenced plants showed homogeneous white areas usually covering whole leaves (**Fig. 4B, 4C**), while in tomato, a patched pattern was observed (**Fig. 4E, 4F**). Rotenberg *et al.* (2006) found photobleaching pattern differences in VIGS experiments which were attributed to unequal virus replication, based on RT-PCR analyses. No differences were observed on photobleaching when either *Sl*PDS o

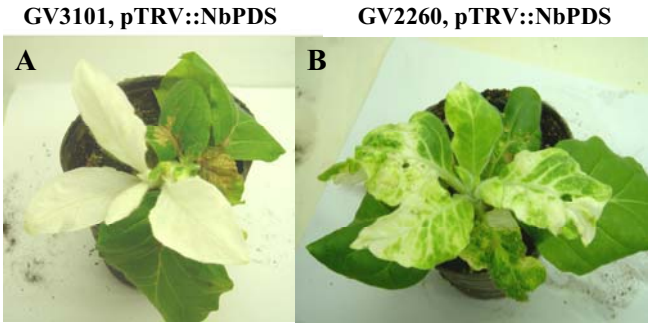

Fig. 5 PDS gene silencing on tobacco plants using different strains. Tobacco plants were agroinfiltrated with (**A**) *A. tumefaciens* strain GV3101 or (**B**) GV2260 carrying TRV-based VIGS vectors system "B" (pTRV1-pTRV2::*Nb*PDS). Pictures were taken 42 dpi.

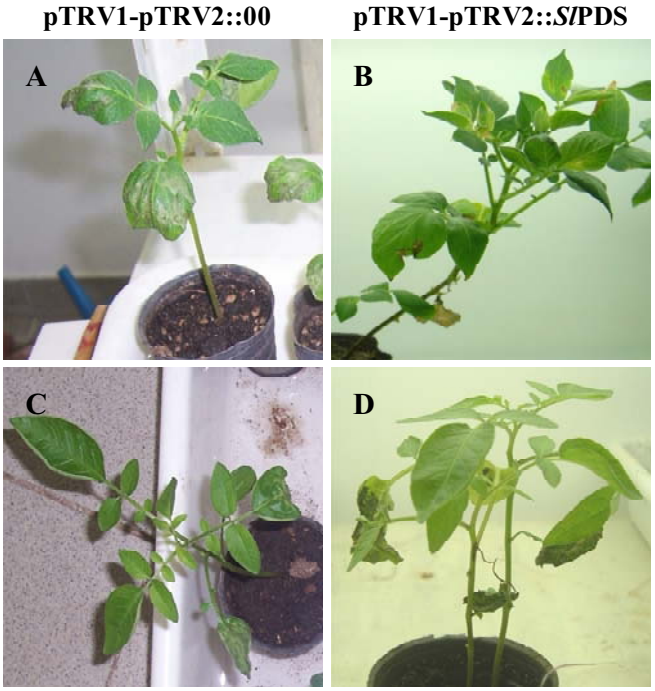

Fig. 6 PDS gene silencing on potato plants. (**A, B**) cultivated potato (cv. 'Bintje', 4X) and (**C, D**) wild potato (*S. chacoense*, 2X). Plants were Agroinfiltrated with *A. tumefaciens* (strain GV3101) carrying TRV-based VIGS vectors: pTRV1-pTRV2::00 TRV system "B" without insert, pTRV1-pTRV2::*Sl*PDS: TRV system "B" with PDS tomato insert. Pictures were taken 35 dpi.

*Nb*PDS was used, most probably as a consequence of the high identity among these sequences. Best results in control plants were achieved when OD_{600} = 0.6 and 4 weeks old plants were used. The used of seedlings or weak plants has been previously recommended by Brigneti *et al*. (2004), however even when working with 2 weeks old small tuber-grown plants photobleaching was not detected. System B produced earlier symptoms than System A (3 vs. 4 weeks after inoculation) in tobacco and tomato plants, probably by the presence of a stronger promoter and a ribozyme that ensures generation of a precise 3′ end of the RNA. Similar results were found by Hart *et al.* (2008) on *S. nigrum*. We have also found that strain GV3101 generates a more homogeneous photobleaching than strain GV2260 (**Fig. 5**), this could be related to the capability of the strain to invade the tissue. Therefore, based in above mentioned results, we chose system "B" and strain GV3101 for following experiments.

Photobleaching was not detected on potato cultivar 'Bintje' (**Fig. 4H**), or any of the other three tetraploid potato cultivars tested, 'Pampeana INTA' (Norero *et al*. 2008), 'Atlantic' and 'Spunta' (unpublished data) nor in any of 10 individuals from a *S. chacoense* diploid population (**Fig. 6**; Saltarini 2007). All these results were confirmed doubling the number of plants (10 plants per genotype and per treatment) in a subsequent independent experiment. It is known that some plant viruses has silencing suppressors (Díaz-Pendón and Ding 2008), however the lack of silencing on *S. tuberosum* plants could not be attributed to silencing suppressors of a previous viral infection because all plant material was originated from virus free *in vitro* plants or seed tubers, and also tested by ELISA for all common potato viruses (PVX, PVS, PVY and PLRV).

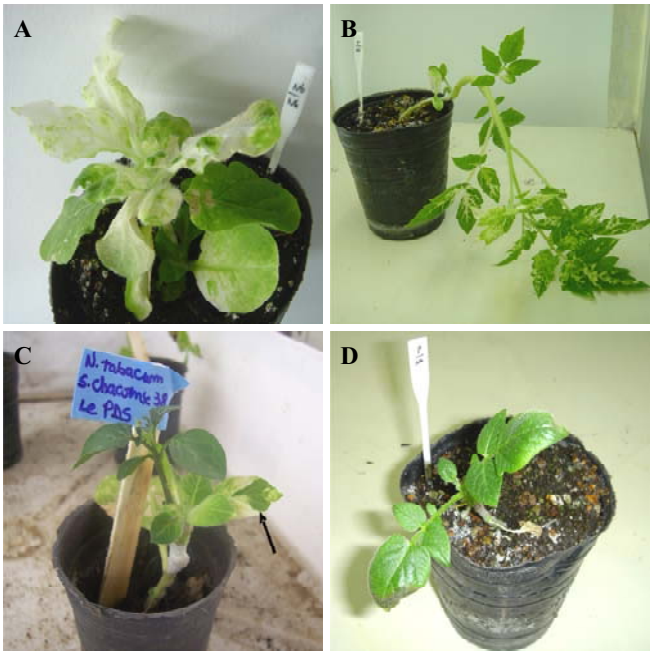

Fig. 7 *Sl*PDS silencing mediated by TRV vector (system "B") on grafted plants. The figure shows grafts, where silenced 4, 5 weeks Agroinfiltrated tobacco plants were used as rootstocks and sealed to different scion plants: (**A**) tomato (positive control), (**B**) tobacco (positive control), (**C**) *S. tuberosum* (cv. 'Pampeana INTA') (4X) and (**D**) *S. chacoense* (2X). Pictures were taken 21 days post grafting. The arrow indicates photobleaching in the silenced tobacco rootstocks.

We have noticed that agroinfiltrated tomato and tobacco leaves suffered necrosis at the inoculation area 3-4 days post infection (dpi), while in potato (both 2X and 4X) necrosis usually extended to whole leaves and petioles, which died at 10 dpi (**Fig. 6**). Phloem necrosis related to virus infection has been described on *Solanum* sp before (Swiezynski *et al.* 1989) and this would reflect the plant defense mechanism to avoid virus dissemination and reduce virus phloem concentration. To discard that absence of silencing in potato could be related to the inoculation method, two alternative techniques were assayed to introduce VIGS vectors: i) agroimmersion and ii) grafting on silenced plants (**Fig. 7B, 7C**). Agroimmersion consists on the immersion of scraped roots of 4-weeks-old tobacco and tomato plants and 2-weeks-old small tuber-grown potato plants in agroinfiltration buffer. It was used buffer alone (control) or buffer carrying System "B" in a RNA1:RNA2 relation of 1:1 (OD_{600} = 0.6) with and without S*l*PDS insert. Plants were agroimmersed overnight at 18°C in the dark and the following day planted at 16-h photoperiod. In tobacco and tomato control plants, photobleaching was observed 2 days earlier than in agroinfiltrated plants (20 dpi). However, a very low efficiency was obtained as only 30% of the treated plant showed symptoms. Again, no silencing symptoms were observed either in 2X or 4X potato plants even two months after treatment (data not shown).

Grafting over transgenic silenced tobacco plants has been first reported by Palauqui *et al.* (1997) to demonstrate the spreading of the RNA silencing signal in stable transformed plants. In our experiments instead of transgenic

Fig. 8 Schematic representation of the PVP-Ar genome and open reading frames (ORFs). The scale shown below indicates the first and final nucleotides for each ORF. RdRp: replication protein, TGBs: triple gene block, CP: capsid protein and NABP: nucleic acid binding protein.

plants, a novel approach using VIGS PDS silenced rootstocks was attempt. Two weeks old scions from 2X (*S. chacoense*) and 4X potato plants (cv. 'Pampeana INTA' and 'Bintje') were grafted on 33 days old tobacco and tomato rootstocks, 3 days post agroinfiltration on two completed expanded lower leaves. Four-weeks-old tomato and tobacco unsilenced scions were used as controls. Grafts were covered for 5 days with a plastic bag and maintained at 18°C in a 16-h photoperiod. Photobleaching was observed on tomato and tobacco scions 2 weeks after grafting (**Fig. 7A, 7B**), even using rootstocks from a different Solanaceous species (i.e. tomato over tobacco and *vice versa*). Contrary to agroimmersion, a high rate of silencing (over 90%) was evidenced on control scions plants (both tomato and tobacco) 21 days post grafting. However, none of the different diploid and tetraploid potato genotypes used in this study (*S. chacoense*; 'Bintje'; 'Pampeana INTA') showed symptoms of silencing (**Fig. 6C, 6D**) even 8 weeks post grafting (Norero *et al*. 2008). Interestingly, **Fig. 7D** shows silenced symptoms on the rootstock but no photobleaching in the 2X potato scion. Moreover, preliminary results of RT-PCR detected TRV-RNA2 (that carries the PDS sequence) in *S. chacoense* apical leaves, indicating that the vector was transmitted but did not work as expected.

After initial reports by Ratcliff *et al.* (2001) and Liu *et al.* (2002), only two additional papers reported successful silencing experiments using TRV - based vectors. Brignetti *et al* (2004) showed silencing of *Sl*PDS and 3 resistance genes related to potato pathogens (Rx, RB, and R1); whereas Hartl *et al* (2008) silenced PDS gene and an aminopeptidase leucine gene in *S. nigrum*. The afore mentioned studies have used plants grown from true seed of *S. okadae* (2X) and *S. nigrum* (6X) and plants grown from small tubers of *S. bulbocastanum* (2X). In the case of *S. tuberosum*, silencing with TRV-based vectors has been reported to work in plants grown from true seeds, with a moderated success. Brigneti *et al.* (2004), reported that only 40% of F1 progeny from cv. 'Cara' (4X) evidenced silencing with System "A". However, because of the clonal nature of potato, seeds from cultivar plants do not share the same genotype as the mother plant (unless a double monoploid is used) hampering the possibility of using duplicates in experiments designed to determine gene function. TRV-based silencing vectors seem a very powerful tool for functional studies, either in tobacco or tomato, as the technique is easy and reproducible. The negative results reported here suggest that TRV-based vectors are not a straightforward method for silencing potato either *S. tuberosum* or *S. chacoense*. The shortage of literature showing successful potato silencing after original papers were published, seems to support this idea. In this sense, we propose to develop a new silencing system for potato.

DEVELOPMENT OF A SPECIFIC POTATO VECTOR FOR VIGS

Recently, our group has obtained the complete genome sequence of potato virus P strain Argentina (PVP-Ar; Massa *et al*. 2008a). PVP-Ar, previously known as *Potato Rough Dwarf Virus* (PRDV) (Butzonitch *et al*. 1996), is a member of *Carlavirus* genus with a positive-strand RNA of 8,404 bases and six open reading frames (ORFs) arranged in a typical genomic organization of the genus (Adams *et al*. 2004; **Fig. 8**).

PVP-Ar presents many features that are desirable in a silencing vector, such as: i) a systemic infection in a wide host range, ii) is asymptomatic in commercial potatoes that could affect interpretation of silenced phenotype iii) a high multiplication rate and accumulation and iv) mechanical transmission (Butzonitch *et al*. 1996; Massa *et al*. 2006). Another indirect indication of the suitability of PVR-Ar is a recent report on the developing of a silencing vector based on *Poplar mosaic virus*, another member of the *Carlavirus* genus, that prove successful in silencing the expression of green fluorescent protein in transgenic plants of *Nicotiana benthamiana* (Naylor *et al*. 2005).

A possible drawback of PVP-Ar is that it shows a putative silencing suppressor in its ORF6, a nucleotide binding protein that presents a SXXXXXXRA motif reported in diverse silencing suppressors (Chiba *et al*. 2006). This possibility has been tested by double infections with PVP-Ar and PVY or PVX, as the activity of a silencing suppressor of one of the virus in the co-infection can be evidenced by the appearance of synergism (Yang and Ravelonandro 2002). Our co-infection results showed that ORF6 of PVP-Ar could be a non-functional or a weak silencing suppressor (Massa *et al*. 2008b). In concordance, Lukhovitskaia *et al*. (2005) reported a similar case in *Chrysanthemum virus B* (CVB), which presents a coding sequence for a nucleic acid binding protein that is involved in infection but does not act as a silencing suppressor.

These results indicate that it is possible to develop a vector of silencing with PVP-Ar without further modifications.

CONCLUSION AND FUTURE PROSPECTS

Entering the Genomics era, Plant Sciences are facing a new revolution that will enable the understanding of the mechanisms underlying many (if not most) of the important trait for crop production. The complete sequence of potato will allow the identification of all the encoded genes. However, the confirmation of their *in planta* role will require additional research that would certainly involve over-expression, complementation or knock-out strategies. Moreover, different allelic contribution to the trait should be determined in order to assess the diversity present in Germplasm Banks. Plant breeding will make use of the superior alleles for a given situation in new improved varieties. Based on the sequence differences found in the allelic variants, ideal molecular markers can be designed for Marker Assisted Breeding (MAB), nonetheless, due to the clonal, outcrossing and tetraploid nature of most of the commercial and traditional potatoes, introgression of superior alleles in existing varieties would be impossible, without the use of genetic engineering. This approach would provide a short term release of better potato varieties; however a change in the public perception of the genetically modified organisms

(GMOs) would be necessary. The use of MAB in potato breeding will be better applied in the early steps of the selection, to reduce the number of initial tested genotypes or to tag the presence of desired alleles for traits that are evaluated late in the breeding process such as "frying quality" or "cold sweetening".

Genetic modification could be applied for end uses different than human consumption like the production of molecules potato with industrial interest. Novel products or increased quantities of existing products in low concentration can be achieved by metabolic engineering of potato plants, through modification of expression of key enzymes of metabolic pathways.

The availability of a vector for transient silencing will greatly help in large scale experiments aimed to identify gene function, and will serve as a rapid screening method to select genes and alleles for later stable transformation events (knock out or overexpression).

The better understanding of the genes involved in secondary metabolism such as P450 family or terpene synthases, between many others, can provide the basis for metabolic engineering to transform potato in a bio-factory for novel molecules with potential use in pharmaceutical, cosmetical or agrochemical industry. In the same manner, potatoes with modified starch via primary metabolism can be used as an "environmentally friendly" alternative to plastics.

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REFERENCES

Adams MJ, Antoniw JF, Bar-Joseph M, Brunt AA, Candresse T, Foster GD, Martelli GP, Milne RG, Fauquet CM (2004) The new plant virus family Flexiviridae and assessment of molecular criteria for species demarcation. *Archives of Virology* **149**, 1045-1060

Alyokhin A (2009) Colorado potato beetle management on potatoes: Current challenges and future prospects. In: Tennant P, Benkeblia N (Eds) *Potato II. Fruit, Vegetable and Cereal Science and Biotechnology* **3 (Special Issue 1)**, 10-19

Andersen JR, Lübberstedt T (2003) Functional markers in plants. *Trends in Plant Sciences* **8**, 554-560

Antão CM, Malcata FX (2005) Plant serine proteases: biochemical, physiological and molecular features. *Plant Physiology and Biochemestry* **43**, 637-50

Atencio M, Clausen A, Izpizúa V, Feingold SE (2008) Variabilidad morfológica y genética en la variedad collareja (*Solanum tuberosum* ssp. *andigena*). *XXIII Congreso de la Asociación Latinoamericana de la Papa*, Mar del Plata, Argentina, proceedings, 143-144

Atencio M, Clausen A, Izpizúa V, Feingold SE (2009) Diversidad genética en la variedad "Collareja" *Solanum tuberosum ssp. andigena*) evaluada con microsatélites. *VII Simposio de Recursos Genéticos Para América Latina y El Caribe*, Pucón, Chile, 297-298

Avula RY, Singh RK (2009) Functional properties of potato flour and its role in product development – A review. In: Yee N, Bussell WT (Eds) *Potato IV. Food* **3 (Special Issue 2)**, 105-112

Baulcombe DC, Chapman S, Santa Cruz S (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *Plant Journal* **7**, 1045-1053

Baulcombe D (1999) Viruses and gene silencing in plants. *Archives Virology Supplement* **15**, 189-201

Blanco M, Valverde R, Gómes L (2003) Optimización de la transformación genética con *Agrobacterium rhizogenes*. *Agronomía Costarricense* **27**, 19-28

Bonierbale MW, Plaisted RL, Pineda O, Tanksley SD (1994) QTL analysis of trichome-mediated insect resistance in potato. *Theoretical and Applied Genetics* **87**, 973-987

Bonierbale MW, Plaisted RL, Tanksley SD (1993) A test of the maximum heterozygosity hypothesis using molecular markers in tetraploid potatoes. *Theoretical and Applied Genetics* **86**, 481-491

Bouché N, Bouchez D (2001) *Arabidopsis* gene knockout: Phenotypes wanted. *Current Opinion in Plant Biology* **4**, 111-117

Bradley JT, Reynolds SH, Weil C, Springer N, Burtner C, Young K, Bowers E, Codomo CA, Enns LC, Odden AR, Greene EA, Comai L, Henikoff S (2004) Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biology* **4**, 12

Brignetti G, Martin-Hernandez AM, Jin H, Chen J, Baulcombe DC, Baker B, Jones JDG (2004) Virus-induced gene silencing in *Solanum* species. *The Plant Journal* **39**, 264-272

Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ, Watson L, Zurcher EJ (1996) Plant viruses online: descriptions and lists from the VIDE database. In: Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ, Watson L, Zurcher EJ (Eds) Available online: http://biology.anu.edu.au/Groups/MES/vide/

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal* **39**, 734-746

Butzonitch IP, Nieto A, Truol GAM, Colavita ML (1996) Características de un nuevo *Carlavirus* relacionado con el virus S de la papa, hallado en Balcarce, Argentina. V Congreso Argentino de Virología y II Encuentro de Virólogos Latinoamericanos. Tandil, Argentina, 16 pp

Chen X, Salimini F, Gebhardt C (2001) A potato molecular function map for carbohydrate metabolism and transport. *Theoretical and Applied Genetics* **102**, 284-295

Chiba M, Reed JC, Prokhnevsky AI, Chapman EJ, Mawassi M, Koonin EV, Carrington JC, Dolja VV (2006) Diverse suppressors of RNA silencing enhance agroinfection by a viral replicon. *Virology* **346**, 7-14

Cogoni C, Macino G (1999) Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* **399**, 166-169

Colman SL, Feingold SE, Monti MC (2009) A microsatellite motif within an invertase gene is associated with cold sweetening in potato. SAIB XLV Annual Meeting. 10-13 November 2009. San Miguel de Tucumán, Argentina. *BIOCELL* **33 (Suppl)**, 136

Demmig-Adams B, Adams WW (1992) Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**, 599-626

Díaz-Pendón JA, Ding S-W (2008) Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annual Review of Phytopathology* **46**, 303-326

Donson J, Kearney CM, Hilf ME, Dawson WO (1991) Systemic expression of a bacterial gene by tobacco mosaic virus-based vector. *Proceedings of the National Academy of Sciences USA* **88**, 7204-7208

Douches DS, Freyre R (1994) Identification of genetic factors influencing chip color in diploid potato (*Solanum* spp). *American Potato Journal* **71**, 581-590

Engel F (1984) *Prehistoric Andean Ecology. Man, Settlement and Environment in the Andes*, Humanities Press, Hunter College, City University of New York

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*. *Molecular Breeding* **6**, 25-36

Faivre-Rampant O, Gilroy EM, Hrubikova K, Hein I, Millam S, Loake GJ, Birch P, Taylor M, Lacomme C (2004) Potato virus X-induced gene silencing in leaves and tubers of potato. *Plant Physiology* **134**, 1308-1316

FAO (2010) Crops statistics database. Available online: http://www.faostat.fao.org/

Feingold SE, Knauber D, Lafta A, Lorenzen J (2004) Mapping P450 genes in potato. In: *Plant and Animal Genome XII Conference*, San Diego, California. USA. P574, p 214

Feingold SE, Lloyd J, Norero N, Bonierbale M, Lorenzen J (2005) Map location and diversity values for new potato SSRs developed from EST databases. *Theoretical and Applied Genetics* **111**, 456-466

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811

Freyre R, Douches DS (1994) Development of a model for marker assisted selection of specific gravity in diploid potato across environments. *Crop Sciences* **34**, 1361-1368

Gebhardt C, Valkonen JPT (2001) Organization of genes controlling disease resistance in the potato genome. *Annual Review of Phytopathology* **39**, 79-102

Ghislain MB, Trognitz B, Herrera Ma del R, 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 *Solanum tuberosum* grown under short-day conditions. *Theoretical and Applied Genetics* **103**, 433-442

Ghislain M, Nuñez J, Herrera MR, Pignataro J, Guzman F, Bonierbale M, Spooner DM (2009) Robust and highly informative microsatellite-based genetic identity kit for potato. *Molecular Breeding* **23**, 377-388

Godge MR, Purkayastha A, Dasgupta I, Kumar PP (2008) Virus-induced gene silencing for functional analysis of selected genes. *Plant Cell Reports*

27, 209-219

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950-952

Hartl M, Merker H, Schmidt DD, Baldwin IT (2008) Optimized virus-induced gene silencing in *Solanum nigrum* reveals the defensive function of leucine aminopeptidase against herbivores and the shortcomings of empty vector controls. *New Phytologist* **179**, 356-365

Hawkes JG (1994) Origins of cultivated potatoes and species relationships. In: Bradshaw JE, Mackay GR (Eds) *Potato Genetics*, CAB, Wallingford, UK, pp 3-42

Huamàn Z, Spooner DM (2002) Reclassification of landrace populations of cultivated potatoes (*Solanum* sect. Petota). *American Journal of Botany* **89**, 947-965

Hull R (2002) *Matthews' Plant Virology* (4th Edn), Academic Press, New York, 1056 pp

Jander G, Barth C (2007) Tandem gene arrays: A challenge for functional genomics. *Trends in Plant Science* **12**, 203-210

Jordá L, Coego A, Conejero V, Vera P (1999) A genomic cluster containing four differentially regulated subtilisin-like processing protease genes is in tomato plants. *The Journal of Biological Chemistry* **274**, 2360-2365

Jordá L, Conejero V, Vera P (2000) Characterization of P69E and P69F, two differentially regulated genes encoding new members of the subtilisin-like proteinase family from tomato plants. *Plant Physiology* **122**, 67-73

Kalendar R, Lee D, Schulman AH (2009) FastPCR software for PCR primer and probe design and repeat search. *Genes, Genomes and Genomics* **3 (Special Issue 1)**, 1-14

Kuhn R, Löw I (1961) Zur Konstitution der Leptine. *Chemische Berichte* **94**, 1088-1095

Kuang H, Wei F, Marano MR, Wirtz U, Wang X, Liu J, Shum WP, Zaborsky J, Tallon LJ, Rensink W, Lobst S, Zhang P, Tornqvist CE, Tek A, Bamberg J, Helgeson J, Fry W, You F, Luo MC, Jiang J, Buell CR, Baker B (2005) The R1 resistance gene cluster contains three groups of independently evolving, type I R1 homologues and shows substantial structural variation among haplotypes of *Solanum demissum*. *The Plant Journal* **44**, 37-51

Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proceedings of the National Academy of Sciences USA* **92**, 1679-1683

Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. *The Plant Journal* **31**, 777-786

Lukhovitskaya NI, Solovyev AG, Koshkina TE, Zavriev SK, Morozov S (2005) Interaction of cysteine-rich protein of Carlavirus with plant defense system. *Molecular Biology (Moskow)* **39**, 896-904

Massa GA, Segretin ME, Colavita M, Riero MF, Bravo-Almonacid F, Feingold SE (2006) Biological and sequence data suggest that potato rough dwarf virus (PRDV) and potato virus P (PVP) are strains of the same species. *Archives of Virology* **151**, 1243-1247

Massa GA, Portantier M, Segretin ME, Bravo-Almonacid FF, Feingold SE (2008a) Comparison of complete sequences of potato rough dwarf virus and potato virus P and their relationships to other carlaviruses. *Archives of Virology* **53**, 1787-1789

Massa GA, Divito SB, Bravo-Almonacid FF, Feingold SE (2008b) En búsqueda de un supresor de silenciamiento en PVP-Ar. XXIII Congreso de la Asociación Latinoamericana de la Papa. Mar del Plata, Argentina, pp 236-237

Matzke MA, Matzke AJM (2000) *Plant Gene Silencing*, Kluwer Academic Press, Dordrecht

Meichtry J, Amrhein N, Schaller A (1999) Characterization of the subtilase gene family in tomato (*Lycopersicon esculentum* Mill.). *Plant Molecular Biology* **39**, 749-760

Menéndez MC, Ritter E, Schafer-Pregl R, Walkemeier B, Kalde A, Salamini F, Gebhardt C (2002) Cold sweetening in diploid potato: Mapping quantitative trait loci and candidate genes. *Genetics* **162**, 1423-1434

Naess SK, Braden JM, Wielgus SM, Haberlach GT, McGrath JM, Helgeson JP (2000) Resistance to late blight in *Solanum bulbocastanum* is mapped to chromosome 8. *Theoretical and Applied Genetics* **101**, 697-704

Napoli CA, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in *trans*. *The Plant Cell* **2**, 279-289

Naylor M, Reeves J, Cooper JI, Edwards M-L, Wang H (2005) Construction and properties of a gene-silencing vector based on *Poplar mosaic virus* (genus *Carlavirus*). *Journal of Virological Methods* **124**, 27-36

Norero N, ten Have A, de la Canal L, Feingold SE (2007) Subtilisinas p69 en papa: Están relacionadas con la respuesta frente a *Phytopthora infestans*? *REDBIO VI Encuentro Latinoamericano de Biotecnología Agropecuaria*. Viña del Mar, Chile, 586:3

Norero NS, Saltarini S, Casalongué C, de la Canal L, Feingold SE (2008) Silenciamiento génico mediado por TRV (*Tobbaco rattle virus*) en *Solanáceas. Abstract in XXXVII Congreso Argentino de Genética*. Tandil, Argentina. *Journal of Basic and Applied Genetics*, p 65

Palauqui JC, Elmayan T, Pollien JM, Vaucheret H (1997) Systemic acquired silencing: Transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non- silenced scions. *EMBO Journal* **16**, 4738-4745

Ratcliff F, Martin-Hernandez MA, Baulcombe DC (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. *The Plant Journal* **25**, 237-245

Rodríguez E, Feingold SE, Lorenzen J, Hu X-J (2007) Análisis genómico de terpeno-sintasas en *Solanum tuberosum*. REDBIO 2007. VI Encuentro Latinoamericano de Biotecnología Agropecuaria. Viña del Mar, Chile, 371:5

Romano N, Macino G (1992) Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Molecular Microbiology* **6**, 3343-3353

Ruiz MT, Voinnet O, Baulcombe DC (1998) Initiation and maintenance of virus-induced gene silencing. *Plant Cell* **10**, 937-946

Rotenberg D, Thompson TS, German TL, Willis DK (2006) Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *Journal of Virology Methods* **138**, 49-59

Ryu CM, Anand A, Kang L, Mysore KS (2004) Agrodrench: A novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. *The Plant Journal* **40**, 322-331

Sagredo B, Lafta A, Casper H, Lorenzen J (2006) Mapping of genes associated with leptine content of tetraploid potato. *Theoretical and Applied Genetics* **114**, 131-142

Saltarini S (2007) Estudios de genes de papa vinculados a estrés fúngico y optimización de la estrategia de VIGS. Graduate thesis, Universidad Nacional de Mar del Plata, Argentina, 54 pp

Schäfer-Pregl R, Ritter E, Concilio L, Hesselbach J, Lovatti L, Walkemeier B, Thelen H, Salamini F, Gebhardt C (1998) Analysis of quantitative trait loci (QTLs) and quantitative trait alleles (QTAs) for potato tuber yield and starch content. *Theoretical and Applied Genetics* **97**, 834-846

Scott KD, Eggler P, Seaton G, Rossetto M, Ablett EM, Lee SL, Henry RJ (2000) Analysis of SSRs derived from grape ESTs. *Theoretical and Applied Genetics* **100**, 723-726

Sijen T, Vijn I, Rebocho A, van Blokland R, Roelofs D, Mol JN, Kooter JM (2001) Transcriptional and posttranscriptional gene silencing are mechanistically related. *Current Biology* **11**, 436-440

Sinden SL, Sanford LL, Cantelo WW, Deahl KL (1986) Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera: Chrysomelidae) in *Solanum chacoense*. *Environmental Entomology* **15**, 1057-1062

Sorensen KK, Madsen MH, Kirk HG, Madsen DK, Torp AM (2006) Linkage and quantitative trait locus mapping of foliage late blight resistance in the wild species *Solanum vernei*. *Plant Breeding* **125**, 268-276

Soderlund C, Humphray S, Dunham A, French L (2000) Contigs built with fingerprints, markers, and FPC V4.7. *Genome Research* **10**, 1772-1787

Swiezynski KM, Dziewonska MA, Ostrowska K (1989) Resistance to the *Potato leafroll virus* (PLRV) in diploid potatoes. *Plant Breeding* **103**, 221-227

Szafranek B, Szafranek J (2008) Volatiles of *Solanum* spp.: Analysis, composition and ecological significance. In: Benkeblia N, Tennant P (Eds) *Potato I. Fruit, Vegetable and Cereal Science and Biotechnology* **2 (Special Issue 1)**, 145-155

Tian M, Huitema E, Cunha L, Torto-Alalibo T, Kamoun S (2004) A Kazal-like extracellular serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B. *The Journal of Biological Chemistry* **279**, 26370-26377

Tian M, Benedetti B, Kamoun S (2005) A second Kazal-like protease inhibitor from *Phytophthora infestans* inhibits and interacts with the apoplastic pathogenesis-related protease P69B of tomato. *Plant Physiology* **138**, 1785-1793

Tornero P, Conejero V, Vera P (1996) Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plants: Similarity of functional domains to subtilisin-like endoproteases. *Proceedings of the National Academy of Sciences USA* **93**, 6332-6337

van Eck H, Jacobs J, Van Dijk J, Stiekema W, Jacobsen E (1993) Identification and mapping of three flower colour loci of potato (*S. tuberosum* L.) by RFLP analysis. *Theoretical and Applied Genetics* **86**, 295-300

van Eck H, Jacobs J, Stam P, Ton J, Stiekema W, Jacobsen E (1994a) Multiple alleles for tuber shape in diploid potato detected by qualitative and quantitative genetic analysis using RFLPs. *Genetics* **137**, 303-309

van Eck H, Jacobs J, Van den Berg P, Stiekema W, Jacobsen E (1994b) The inheritance of anthocyanin pigmentation in potato (*Solanum tuberosum* L.) and mapping of tuber skin colour loci using RFLPs. *Heredity* **73**, 410-421

van Os H, Andrzejewski S, Bakker E, Barrena I, Bryan GJ, Caromel B, Ghareeb B, Isidore E, de Jong W, van Koert P, Lefebvre V, Milbourne D, Ritter E, van der Voort JN, Rousselle-Bourgeois F, van Vliet J, Waugh R, Visser RG, Bakker J, van Eck HJ (2006) Construction of a 10,000-marker ultradense genetic recombination map of potato: Providing a framework for accelerated gene isolation and a genomewide physical map. *Genetics* **173**, 1075-1087

Vaucheret H, Beclin C, Fagard M (2001) Post-transcriptional gene silencing in plants. *Journal of Cell Science* **114**, 3083-3091

Varshney RK, Graner A, Sorrells ME (2005) Genic microsatellite markers in plants: Features and applications. *Trends in Biotechnology* **23**, 48-55

Visser RGF, Bachem CWB, de Boer JM, Bryan GJ, Chakrabati SK, Feingold SE, Gromadka R, van Ham RCHJ, Huang S, Jacobs JME, Kuznet-

sov B, de Melo PE, Milbourne D, Orjeda G, Sagredo B, Tang X (2009) Sequencing the potato genome: Outline and first results to come from the elucidation of the sequence of the world's third most important food crop. *American Journal Potato Research* **86**, 417-429

Xu D-Q, Huang J, Guo S-Q, Yang X, Bao Y-M, Tang H-J, Zhang H-S (2008) Overexpression of a TFIIIA-type zinc finger protein gene ZFP252 enhances drought and salt tolerance in rice (*Oryza sativa* L.). *FEBS Letters* **582**, 1037-1043

Yang S, Ravelonandro M (2002) Molecular studies of the synergistic interactions between plum pox virus HC-Pro protein and potato virus X. *Archives of Virology* **147**, 2301-2312

Zhao Y, Thilmony R, Bender CL, Schaller A, He SY, Howe GA (2003) Virulence systems of *Pseudomonas syringae* pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. *The Plant Journal* **36**, 485-499