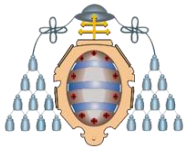

Universidad de Oviedo

Programa de Doctorado:

Biotecnología alimentaria

Uso de marcadores moleculares en mejora y trazabilidad en patata (*Solanum tuberosum*)

Claudia López Vizcón

Abril 2015

AGRADECIMIENTOS

Mi agradecimiento infinito a mi tutora y co-directora, la Dra. Eva García, por ser mi tutora y mucho más en este viaje. Por su entusiasmo sin fin, su ayuda y consejos, su dedicación, sus conocimientos… Y por los momentos en que tutoraste también a la persona, no sólo a la doctoranda.

A mi otra co-directora, la Dra. Felisa Ortega, por recorrer conmigo un camino que no fue fácil, pero que terminamos, a pesar de todo y de todos. Aprendí mucho recorriéndolo, ese es el mejor regalo.

A los miembros del laboratorio del Área de Genética de la Facultad de Medicina, en especial, al Dr. Yaisel Borrell, por acogerme, ayudarme y aconsejarme.

A mis compañeros en la empresa APPACALE, en la que desarrollé casi todo el trabajo que ha dado origen a esta Tesis. Espero que la sientan un poco suya, y que vean aquí un pequeño homenaje al trabajo y la dedicación de unos burgaleses que me enseñaron mucho.

A mis biólogos, especialmente a Patri, Paula, Óscar, Javo y Mike. Sois de lo primero y lo mejor que me dio la Biología. Sólo por eso, mereció la pena.

A otros biólogos y no biólogos, que fui encontrando en el camino, en ese que pasó por Holanda y por Pamplona. Por los momentos de gloria y de pánico en el laboratorio. Y por todos los demás. A Edu y Guti, que traspasaron pronto la barrera de compañeros. Por cuidarme mucho siempre. Y a Maite y María, por cuidarme tanto como ellos.

A mis amigos.

A mis amigos de casa, de Asturias, por estar siempre cuando necesito volver a encontrarme frente a su mar, o entre sus montañas. En especial a mis canguesinas, las que están desde siempre y que siempre estarán.

A los burgaleses de adopción, y al único de verdad, por hacer que aquella etapa que fue dura a veces, tuviese sentido.

A toda mi gente de Leiden, con la que compartí uno de los mejores capítulos de mi vida.

A toda mi familia en Madrid, que me recogió en el momento más difícil y no me dejó nunca más. Muy en especial a todos mis héroes en la capital, Ángel, Dani, Ise, Quique, Martina, Álvaro, Zeus y, cómo no, Gonzalo, que me ayudó a escuchar la música cuando llegaba a la meta. Y a la colonia charra, que adoptaron sin pensarlo a esta asturiana.

A mi gente de Leiden en Madrid, Bea, Noe y Stefano. Por estar incondicionalmente.

A Mayka, por ser mi red.

A Patty y Marta, por ser mis personas, por no tener a veces ni que hablar para que entendáis. A Lèa y Julia por venir a poner luz.

A Esther, porque si hay alguien que ha empujado conmigo desde el principio, en exámenes, experimentos, viajes, estancias, congresos, tesinas… Esa eres tú. Si hay alguien que sabe cómo me siento mientras escribo las líneas finales de esta tesis, eres tú. Por eso sé que entiendes la dimensión de este GRACIAS.

A toda mi familia.

A mi hermano, por darme sin pretenderlo lecciones de coraje y superación.

A mis padres, por todo y por mucho más. Por ser mi ejemplo y mi fuerza.

A mis padres

Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.
Marie Curie

Do not judge me by my successes,
judge me by how many times I fell down and got back again
Nelson Mandela

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1 ABSTRACT

The potato (*Solanum tuberosum*) is the fourth most important crop in the world. In 2012, more than 365 million tons of potatoes were produced worldwide. Europe, which had historically been at the forefront of global producers, lost for the first time this position against emerging powers like China. However, Europe still holds one of the largest consumption per capita in the world. The potato is part of the staple diet of Europeans, and also Spanish. However, the potato sector has suffered a severe setback in the last years in Spain, and most potatoes consumed in our country are imported. Despite this "potato crisis", the crop remains one of the most important in the country. There are numerous varieties of potato available in the market and most of them are classified depending on their taste, quality and end-use. Its price also depends on these features. Under current EU legislation, potatoes offered for sale should be labeled with the name of the variety. This is both for consumer protection and quality controls, and therefore, the development of an effective method of varietal identification is required. The traditional morphological identification methods have proven to be not very effective and reproducible since they can be influenced by different factors, like environmental ones. Moreover, genetic markers provide a reproducible and accurate method of varietal identification.

Despite the large number of varieties available in the market, there is still a need for new cultivars. The potato industry in the European Union is trying to increase potato handling in an economically and environmentally sustainable way. The development of new varieties should provide economic benefits through increased performance due to lower production cost, with fewer attacks of diseases and pests and tolerance to environmental stresses. Developing a new potato variety can take up to 12 years from initial crosses until its release, so improved breeding strategies are needed. Molecular markers offer a striking promise for plant breeding.

RESUMEN

La patata (*Solanum tuberosum*) es el cuarto cultivo más importante del mundo. En 2012, se produjeron en todo el mundo más de 365 millones de toneladas de patata. Europa, que había estado históricamente a la cabeza de los productores mundiales, pierde por primera vez este puesto frente a potencias emergentes como China. Sin embargo, Europa sigue ostentando uno de los mayores consumos per cápita del mundo. La patata forma parte de la dieta básica de los europeos, y también de los españoles. Sin embargo, el sector de la patata ha sufrido un duro retroceso en los últimos años en España, y la mayoría de las patatas consumidas en nuestro país, son importadas. A pesar de esta "crisis de la patata", el cultivo sigue siendo uno de los de mayor importancia a nivel nacional. Existen numerosas variedades de patata disponibles en el mercado y la mayoría se clasifican dependiendo de su sabor, calidad y uso final. Su precio depende también de estas características. Bajo la actual legislación de la UE las patatas que se ponen a la venta deberían etiquetarse con el nombre de su variedad. Esto responde tanto a la protección al consumidor como a los controles de calidad, y por tanto, se hace necesario el desarrollo de un método eficaz de identificación varietal. Los métodos tradicionales de identificación morfológica han demostrado ser poco eficaces y reproducibles al estar influenciados por distintos factores, como los ambientales. Por otra parte, los marcadores genéticos ofrecen un método reproducible y preciso de identificación varietal.

A pesar del gran número de variedades disponibles en el mercado, todavía existe la necesidad de nuevos cultivares. La industria de la patata en la Unión Europea está intentando aumentar el manejo de la patata de un modo sostenible económica y medioambientalmente. El desarrollo de nuevas variedades debería ofrecer beneficios económicos a través de un mayor rendimiento, gracias a un menor coste de producción, con menos ataques de enfermedades y plagas y tolerancia a estreses ambientales. Desarrollar una nueva variedad de patata puede llevar hasta 12 años, desde los cruces iniciales hasta su lanzamiento, así que es necesario perfeccionar las estrategias de mejora. Los marcadores moleculares ofrecen una notable promesa para la mejora genética de plantas.

2. INTRODUCTION

2 INTRODUCTION

2.1 Molecular markers applications to crop production and control

Genetic markers are DNA sequences with a known physical location on a chromosome. They represent genetic differences between individual organisms or species. Normally, they do not represent the target genes themselves; instead they are usually located very close to the gene of interest and so, they can be used for signaling the real target (Collard *et al.*, 2005). Genetic markers can be classified into 3 major categories: (1) morphological (also 'classical' or 'visible') markers which themselves are phenotypic traits or characters; (2) biochemical markers, which include allelic variants of enzymes called isozymes; and (3) DNA (or molecular) markers, which reveal sites of variation in DNA (Winter and Kahl, 1995; Jones *et al.*, 1997). Both morphological and biochemical markers have been extremely useful to plant breeders (WeedResistance to potato virus Y in Solanum tuberosum spen *et al.*, 1993; Eagles *et al.*, 2001). However they may be limited in number and are influenced by environmental factors or the developmental stage of the plant (Winter and Kahl, 1995). Unlike these two types of markers, DNA markers do not present any of these limitations. They arise from different classes of DNA mutations such as substitution mutations (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA (Paterson, 1996). These markers are selectively neutral because they are usually located in non-coding regions of DNA. Apart from the use of DNA markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within germplasm and cultivar identity (Weising, K., H. Nybom, 1995; Winter and Kahl, 1995; Baird, W.V., R.E. Ballard, 1997; Henry, 1997; Jahufer, M., B. Barret, 2003).

2.1.1 Marker-assisted selection

Selecting plants in a segregating progeny that contain appropriate combinations of genes is a critical component of plant breeding (WeedResistance to potato virus Y in Solanum tuberosum spen *et al.*, 1993; Ribaut and Betrán, 1999) Moreover, plant breeders typically work with hundreds or even thousands of populations, which often contain large numbers (Ribaut and Betrán, 1999; Witcombe, 2001). 'Marker-assisted selection' (also 'marker-assisted

breeding' or 'marker-aided selection') may greatly increase the efficiency and effectiveness in plant breeding compared to conventional breeding methods. Once markers that are tightly linked to genes or QTLs of interest have been identified, prior to field evaluation of large numbers of plants, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs (Michelmore, 1995; Young, 1996; Ribaut *et al.*, 1997). Advantages of MAS compared to classical breeding are many: time saving from the substitution of complex field trials with molecular tests; elimination of unreliable phenotypic evaluation associated with field trials due to environmental effects; selection of genotypes at seedling stage; gene 'pyramiding' or combining multiple genes simultaneously; avoid the transfer of undesirable or deleterious genes ('linkage drag'; this is of particular relevance when the introgression of genes from wild species is involved); selecting for traits with low heritability; testing for specific traits where phenotypic evaluation is not feasible (Collard *et al.*, 2005).

One step further, new technologies like plant transformation introducing alien DNA try to overcome classical breeding problem and to enrich the cultivated genetic pool (Tester and Langridge, 2010). However, several issues are likely to limit the application of these new methods, particularly for breeding programs in the public sector. Regulatory complexity and high costs have prevented the widespread delivery of GM technologies. Over the coming decade or so, however, it seems inevitable that GM technologies will become much more widely used—it is probably a case of "when," not "if." A consequence emerging for crops that are now dominated by GM varieties (such as cotton, soybean, and maize) is that breeding programs are now based around GM varieties, and consequently, breeding programs in non-GM jurisdictions have limited access to current advances. The key limitations for traditional breeding include lack of resources, training, and capabilities for most of the world's crop improvement programs.(Godfray *et al.*, 2010) It is important, therefore, that we expand the scope of and access to new marker platforms to provide efficient, cost-effective screening services to the breeders. Communication and mechanisms for delivery of material to breeders must be developed. There is an urgent need to expand the capacity of breeding programs to adopt new strategies. The clearly documented high rate of return on such investments in the past should be kept in mind (Alston *et al.*, 2000) The concerns about food security and the likely impact of environmental change on food production have injected a new urgency into accelerating the rates of genetic gain in breeding programs. Further technological developments are essential, and a major challenge will be to also ensure that the technological advances already achieved are effectively deployed.(Tester and Langridge, 2010)

2.1.2 Traceability and Quality Control

Food safety concerns are not only limited to the breeding process, food processing and labeling are also important issues on food and feed quality and control. Consumers require clear and accurate information to make informed choices about their diet and the foods they buy. The information given to consumers is essential for them choosing one food product over another. Consumer choice might also reflect lifestyle or religious concerns (e.g. vegetarianism, preference for organic products, absence of pork for Jews and Muslims), or health concerns (e.g. absence of peanuts, lactose or gluten for individuals with particular allergies). Therefore, the description and/or labeling of food must be honest and accurate, particularly if the food has been processed removing the ability to distinguish one ingredient from another. The information that must be given is enshrined in law in most developed countries, so that food supplied must be exactly what the labeling says it is. That is, the food must be authentic and not misdescribed (Woolfe and Primrose, 2004).

Traceability is defined as a system able to maintain a credible custody of identification for animals or animal products through various steps within the food chain, from the farm to the retailer (McKean and McKean, 2001). Many different chemical and biochemical techniques have been developed for determining the authenticity of food and in recent years methods based on DNA analysis have become more important (Woolfe and Primrose, 2004). Once more, this is because molecular methods have proven to be better and more reliable tools than other techniques. Immunoassays, for example, work well with raw foods but lose their discrimination when applied to cooked or highly processed foods. Also many techniques do not easily distinguish between closely related materials at the chemical level. Conventional chemical methods are also not always able to detect country or region of origin of raw material. DNA analysis has discriminating power because ultimately the definition of a variety or species is dependent on the sequence of the DNA in its genome. DNA is more resilient to destruction by food processing (particularly cooking and sterilization) than other marker substances(Woolfe and Primrose, 2004). So, molecular markers have become a powerful tool for food and feed fingerprinting and (Bhaskar *et al.*, 2009; Galimberti *et al.*, 2013)

The necessity for labeling of food derived from genetically modified organisms (GMOs) to comply with the Novel Food Regulation (EC/258/97, EC/ 1139/98, EC/49/2000, EC/50/2000 and EC/ 1829/2003) has been updated continuously in European countries (Tung Nguyen *et al.*, 2008). Therefore, the demand for the establishment and development of a robust and rapid operation procedure for GMO detection has increased recently in these countries. Molecular

tools also appear to be the better choice to detect undeclared GMOs and (Tung Nguyen *et al.*, 2008; Holst-Jensen *et al.*, 2012).

2.2 Potato Biology and Origin

The potato (*Solanum tuberosum*) belongs to the *Solanaceae* family. This family includes, among 2000 other species, the tomato (*Lycopersicum esculentum*), sweet pepper (*Capsicum annuum*), eggplant (*S. melongena* var. *esculentum*), tobacco (*Nicotiana tabacum*), and petunia (*Petunia hybrida*). The genus *Solanum* is a polymorphous and largely tropical and subtropical genus containing more than 1000 species (Fernald, 1970)

The potato plant (Fig.1.1.) has weak stems that grow to a maximum of three feet, long pinnate leaves, ovate leaflets with smaller ones disposed along the midrib. The flowers are white, purple, pinkish, or bluish, in clusters, usually with a five parted corolla and exerted stamens with very short filaments. Some varieties are male sterile, suffer from abscission of flowers, and rarely produce fruits. The fruits are yellowish or green, globose, less than one inch in diameter, some lack seeds, but others may contain several hundred. The fruits are inedible for humans, due to the presence of toxins. *Solanum* species have an initial chromosome number of 12, but polyploidy is prevalent in both wild and cultivated potatoes. (Liberty Hyde Bailey Hortorium, 1976)

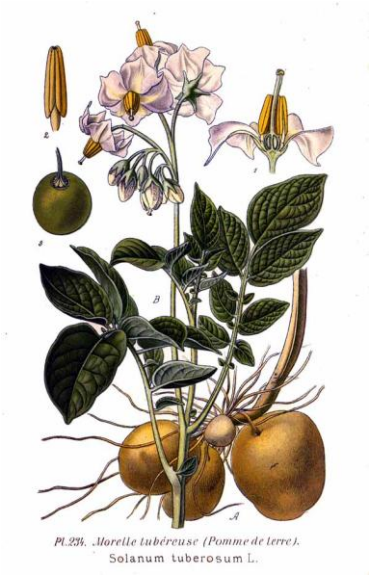

Fig.1.1. *Solanum tuberosum* L. Planta de patata.

The potato's story begins about 8,000 years ago near Lake Titicaca, which sits at 3,800 m (12,500 ft) above sea level in the Andes mountain range of South America, on the border between Bolivia and Peru (Hoopes R.W. & Plaisted R.L., 1987). Almost 200 species of wild potatoes are found all through the American continent, but it was in this particular region where the potato was domesticated and where the largest genetic variety of wild and cultivated species can still be found (Fig.1.2.) (Morales, 2007).There are two major, only slightly different, subspecies of *Solanum tuberosum*; *andigena* or Andean, and *tuberosum* or Chilean (Raker and Spooner, 2002) the potato now cultivated around the world, which is believed to be descended from a small introduction to Europe of *andigena* potatoes that later adapted to longer day lengths(FAO, 2008). The Andean potato is adapted to the short-day conditions prevalent in the equatorial and tropical regions where it originated (Raker and Spooner, 2002). It is indigenous to Andean region from Venezuela to northern Chile and Argentina (Hawkes, 1990).The Chilean potato is adapted to the long-day conditions prevalent in the higher latitude region of southern Chile, especially Chiloé Island and Chonos Archipelago, where it is thought to have originated (Hawkes, 1990; Hijmans, 2001).

Fig.1.2. Different native potato varieties. Image courtesy International Potato Center (Peru).

2.3 Economic and Social Importance of the Potato Crop

Potato (*S. tuberosum*) is the fourth most important crop in the world and a staple part of many diets in several countries. In 2012, more than 365 million tons of potatoes were produced worldwide (FAOSTAT, 2013), more than 70 million tons only in the EU (FAOSTAT, 2013). Europe had been historically the world's biggest producer of potatoes, but FAO data show that in 2005, for the first time, the developing world's potato production exceeded that

of the developed world (FAO, 2008). So, the rapid expansion occurred over the past few decades in southern and eastern Asia has made China the largest world potato producer since 2007. Still, the highest consumption is found in Europe, with almost 90 kg per capita per year (FAO, 2008).

2.3.1 The situation in Spain

In Spain, entry point of the potato in Europe around the mid-1500s (FAO, 2008), the *potato* was a mainstay of Spanish agriculture through most of the 20th century, with annual production exceeding 5 million tones up to the 1990s (FAO, 2008). However, as in other European countries, potato production has decreased dramatically in the past years (FAO, 2008) and current production is around 2,3 million tons per year (MAGRAMA, 2013). Moreover, Spain has become a major potato importer, being 95 % of these imports from UE countries, mainly from France (72% of the imports), U.K., The Netherlands and Belgium (MAGRAMA, 2011). Despite this "potato crisis" in Spain, there are still areas where the potato remains an important crop. The main production area is Castilla y León, responsible for the 40% of annual Spanish production (873.847 Tm/year), presenting a higher crop yield than the national average (JCyL, 2015a). Castilla y León is also the most important area for seed potato production, with more than 70% of the Spanish production (JCyL, 2015b) and the most important seed potato companies are also located in this region.

2.4 Potato Genetics

The cultivated potato (*S. tuberosum* subsp. *tuberosum*) is a tetraploid species (2*n* = 4*x* = 48) which displays tetrasomic inheritance. It was derived from a narrow genetic base of a few introductions of subsp. *andigena* from South America in the late 16th century and possible further introductions in the 17th and 18th centuries (J. E. Bradshaw and G. R. Mackay, 1994). As a consequence, it lacked genes for adequate levels of resistance to pathogens and pests such as late blight (*Phytophtora infenstans* (Mont.) de Bary) or potato cyst nematodes (PCN) (*Globodera* spp.) which became problems once it had assumed as a staple food crop (J. E. Bradshaw and G. R. Mackay, 1994). During the 201th century, several attempts were made to

remedy these deficiencies by the introgression of resistance genes into subsp. *tuberosum* from the wild and cultivated species of central and South America (Malcomson, 1969; Hermsen, 1994; Bradshaw, J.E., Stewart, H.E., Wastie, R.L., Dale, M.F.B. and Phillips, 1995). However, most of the major genes studied failed to give durable resistance to the pathogen (Malcomson, 1969) or resulted to be effective only against determined populations/pathotypes of the pathogen species. In other cases, only partial resistance could not be achieved in successful cultivars, or as a consequence of widespread deployment of a major gene against a determined pathotype, another one was inadvertently selected (Meyer *et al.*, 1998)

2.5 Potato Breeding

Conventional plant breeding is primarily based on phenotypic selection of superior individuals among segregating progenies resulting from hybridization. Classical potato breeding to develop a new potato variety is a long procedure. As described by Tai & Young, 1984, a conventional breeding scheme would have the following steps: as the first step, parents are selected for their potential to produce new and desirable genotypes. True seed derived from the selected parents is then obtained. A large number of seedlings (genotypes), sometimes up to several hundred thousands annually in large breeding programs, are raised from the true seed and evaluated in the seedling (F1) or first clonal generation (Fig. 1.3.). A proportion of these seedlings is selected and replanted in small unreplicated experiment units for screening in the next clonal generation. Accurate testing of advanced clones can be started only after the population has been reduced to a manageable size. Superior lines are promoted into trials of various types (yield, disease resistance, quality, management, etc.) and assessed for merit as potential new varieties (Fig. 1.4.). Conventional potato breeding takes long (about 10 years) before a cultivar is released, mostly due to the slow multiplication rate of the crop (Muthoni *et al.*, 2012).

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Fig.1 3. Seedling (Appacale, S.L., Burgos, Spain

Fig.1.4. Field trial (Palencia, Spain)

2.5.1 Molecular Markers and Potato Breeding

It soon became clear the need to combine quantitative resistance available in subsp. *tuberosum* with commercially acceptable yields and qualities demanded by the industry. Many works on genetical linkage analysis in tetraploid potato have been reported so far, as a prerequisite to molecular-marker-assisted selection (Bradshaw *et al.*, 1998).

Despite the large number of new potato (*Solanum tuberosum* L.) cultivars currently available, there is still a need for new ones. The potato industry in the European Union is trying to increase potato handling in an economically and environmentally sustainable way. New cultivars must give economic benefits through more yield of saleable product at less cost of production, with reduced disease and pest attacks, and tolerance to environmental stresses. Developing a new variety can take up to 12 years from crossings to release, so improved breeding strategies are needed. Molecular markers offer a striking promise for plant breeding. The opportunity to select desirable lines based on genotype rather than phenotype, analyzing plants at the seedling stage, screening multiple characters, minimizing linkage drag and rapidly recovering a recurrent parent's genotype are just some of the attractions of Marker-Assisted Selection (MAS;(Collard and Mackill, 2008). However, it soon became apparent that applying knowledge gained through molecular mapping in real world breeding might not be entirely straightforward. The reality is that MAS has had only a limited impact on plant breeding so far (Young, 1999; Collard and Mackill, 2008). Pests and diseases are the major threat to potato cultivation worldwide. Thus, durable disease and pest resistance is a primary goal of most potato breeding schemes. Classical breeding for resistance involves the identification of

resistance sources, which are often found in wild and unadapted germplasm. Since 1990, many of these resistance factors have been located on the potato molecular linkage map using DNA-based markers. They have been mapped either as major genes (R genes) or as quantitative trait loci (QTL; (Gebhardt and Valkonen, 2001; Simko *et al.*, 2007). The dominant gene for extreme resistance to Potato virus Y (PVY), Ryadg, was identified in S. tuberosum ssp. andigena (Muñoz, F.J. Plaisted, R.L. Thruston, 1975) and mapped to chromosome XI (Hamalainen *et al.*, 1997; Kasai *et al.*, 2000) developed RYSC3 polymerase chain reaction (PCR)-based marker that simultaneously functions for Ryadg and the gene Naadg of hypersensitive resistance to Potato virus A (PVA). Some years later, (Song *et al.*, 2005) mapped the Rysto gene to chromosome XII cosegregating with the molecular marker STM0003. Regarding potato cyst nematodes (PCN), the monogenic dominant Gro1 gene from Solanum spegazzinii for resistance to all pathotypes of Globodera rostochiensis was mapped to the potato molecular map on chromosome VII (Barone *et al.*, 1990). Gro1-4, a member of Gro1 locus, showed to confer resistance to G. rostochiensis pathotype Ro1 (Paal *et al.*, 2004). Besides, the most widely used dominant gene of resistance to G. rostochiensis Ro1 is gene H1 from S. tuberosum spp. andigena, mapped to chromosome V (Gebhardt *et al.*, 1993). On the other hand, the most prominent and reproducible QTL for G. pallida resistance was mapped to chromosome V, and the diagnostic value for the linked DNA marker SPUD1636 was demonstrated by Bryan et al. (Bryan *et al.*, 2002) in some accessions with Solanum vernei as source of resistance. Later on, Sattarzadeh et al. (Sattarzadeh *et al.*, 2006) reported a PCR assay "HC" with higher diagnostic value for this QTL. Disease resistant genotypes are often simple and oligogenic in nature, but the difficulties in establishing reliable inoculation methods and scoring can be a discouraging handicap. MAS can be extremely powerful in this field. But even so, published examples of the use of molecular markers for MAS of disease/pest resistance are mainly limited to diploid material and a small number of genes (Barone *et al.*, 1990; Ottoman *et al.*, 2009).

2.6 Potato Quality and Traceability

Potatoes are consumed in Spain in very different ways: boiled, fried, baked, roasted, and as the main ingredient of the Spanish omelette. Nowadays, there is a large number of potato varieties available in the market and most of them are classified depending on their taste, quality or end-use (Fig.1.5.). Their prizes also depend on those characteristics (Fig.1.6.). Under current EU legislation there is a requirement for potatoes that are offered for wholesale

or retail sale to be labeled with their variety name (Woolfe and Primrose, 2004). This is both for consumer protection and quality control. The general concern about healthy products and specially the competitiveness between producers have made essential the development of a reliable method of varietal identification (Woolfe and Primrose, 2004).

Fig.1.5. Different commercial potato varieties.

Traditionally, morphological traits such as leaf type, tuber shape or flower color have been used to identify potatoes. However, as said before, these traits can be influence by many factors, like environmental ones, making this identification method lack in reliability and reproducibility (Woolfe and Primrose, 2004; Rosa *et al.*, 2010). Moreover, once the potato is processed, varietal identification based on morphological traits will be extremely difficult, when not impossible. Other methods are based on isozymes and total protein extraction (Douches, D. S., & Ludlam, 1991). Again, the results can be influenced by different factors, in this case, development stage and growth conditions of the plant.

Fig.1.6. Different commercial potato varieties with different prizes at a supermarket.

2.6.1 Molecular markers and potato traceability

There are also molecular methods of varietal identification. Random amplified length polymorphisms (AFLP) (Kim, J. H., Joung, H., Kim, H. Y., & Lim, 1998)) and short sequence repeat (SSR) or microsatellite markers (Norero *et al.*, 2002; Ghislain *et al.*, 2004; Moisan-Thiery *et al.*, 2005; Mathias, M., Sagredo, B., & Kalazich, 2007) are the most common ones and have shown to be the most reproducible and reliable. The use of random amplification of polymorphic DNA (RAPD) markers is well suited to DNA fingerprinting (Thormann *et al.*, 1994)but difficulties in the reproducibility of the technique are a limiting factor for accurate analysis (Demeke *et al.*, 1997; Mondini *et al.*, 2009) Microsatellite markers are not only reproducible and reliable, but very informative and simple to use, the method can be automated, and effective cost per genotype and analysis is lower than in other molecular marker technologies (Garcia *et al.*, 2004; Moisan-Thiery *et al.*, 2005). All these reasons justify DNA-based fingerprinting using SSRs as a reliable and efficient method for potato cultivar identification. Despite EU and national legislations on food quality and control, some fraud and mislabelling problems have been detected in the Spanish food industry in the last years, especially in the fish market (Asensio *et al.*, 2008; Garcia-Vazquez *et al.*, 2011). However, the mislabelling levels for vegetables like potatoes is still quite unexplored.

2.7 Genetically Modified Potatoes

Genetically engineered or genetically modified (GM) plants, hereafter referred to as GM organisms (GMOs), are defined as organisms “in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination” (European Comission, 2003). As from the early developments of genetic engineering, the scientific society raised its concerns about this novel technology (Berg, P. Baltimore, D. Brenner, S. RoblinIII, R.O. Singer, 1975). As such, the development and in particular any commercial use of GMOs are subject to strict legal regulations in most countries around the world. The established legal framework mainly addresses all risks for the release of GMO in the environment and for consumption of GM materials for human and animal health. There can also be limitations to the use; a GMO can for example be legally used as a feed but not as a food. To support the freedom of choice of consumers, in many countries a mandatory or voluntary labeling of products containing GM material has been established, including

specifications of thresholds for labeling (Holst-Jensen *et al.*, 2012). EU regulations are very clear on what it concerns to GMO labelling. So, it is a legal requirement that food products where the level of approved GMO exceeds 0.9% of unintentional adventitious presence are appropriately labeled (European Comission, 2003). Once more, molecular markers have been reported as very useful tools for detecting and identifying GMOs (Tung Nguyen *et al.*, 2008; Holst-Jensen *et al.*, 2012).

Only one commercial GM potato has been released and approved in the EU, and only for industrial purposes. Amflora variety, also known as also known as **EH92-527-1**) is a genetically modified potato cultivar developed by BASF Plant Science. 'Amflora' potato plants produce pure amylopectin starch that is processed to waxy potato starch (Oreword and Art, 2011). Amflora™Potato was modified to produce less amylose starch and thus a higher content of amylopectin. This was done through anti-sense suppression of the granule bound starch synthase (GBSS) protein (see Fig.1.7.). GBSS enzyme is one of the key enzymes in the biosynthesis of starch and catalyses the formation of amylose. When the gene is inactivated through antisense technology, the starch produced has little or no amylose and consists of branched amylopectin, which modifies the physical properties of the starch and is advantageous for the starch processing industry (see Fig. 1.8.).

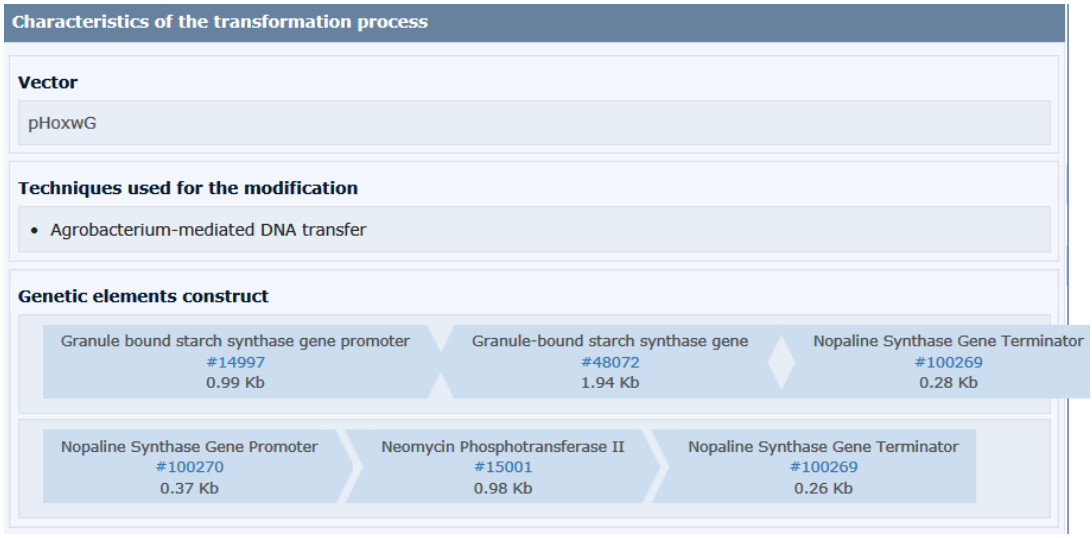

Fig. 1.7. Amflora transformation process data.

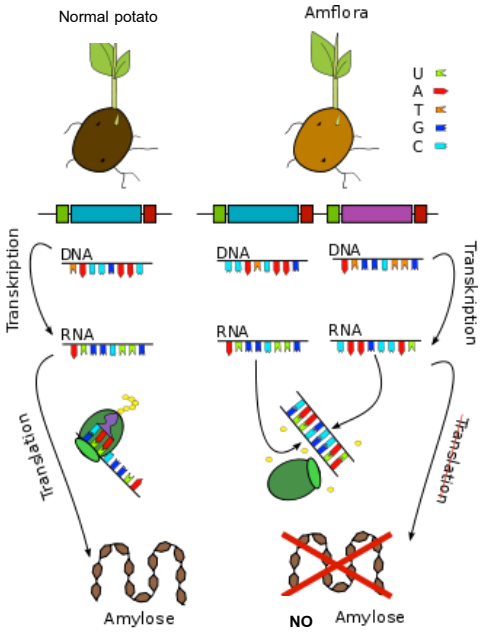

Fig.1.8. Amflora's mechanism for pure-amylopectin starch production.

Despite the fact that only this variety has been approved to date, transgenic potatoes keep being developed and tested (Missiou *et al.*, 2004; Green *et al.*, 2012; Teagasc, 2012), so a good and reliable method for GM potato traceability is also needed.

3. OBJECTIVES

3 OBJECTIVES

Regarding the use of Molecular Markers in Potato Breeding the objectives were:

- To evaluate available molecular markers in a real Potato Breeding Program.
- To compare the effectiveness and costs between Marker Assisted Selection and Classical Breeding methodologies in a real Potato Breeding Program.
- To evaluate the usefulness of molecular markers to calculate the segregation ratios of parental in the Breeding Program.

Regarding the use of Molecular Markers in Traceability, the objectives were:

- To implement an easy-to-use method for potato traceability using molecular markers
- To study fraud levels and mislabeling in the potato industry in Spain
- To study the potential presence of non-declared transgenic potatoes in the Spanish market

4. MATERIAL AND METHODS

4 MATERIALS AND METHODS

4.1 Experiments and Plant Material

4.1.1 Breeding Programme

Breeding clones evaluated for the presence of molecular markers were those of the breeding programme developed at the company which had as parent one or more resistant cultivars known to amplify any of the specific markers described later or a first backcross of these parents. Therefore Ryadg genotypes used were: varieties 7XY- 1, LT-8, LT-9, P-6, Tacna and V-2 and breeding clones 95APP-4 (Spunta × V-2), 95APP-5 (Atlantic × V-2), 95P17-3 and 95P17-7 (Iroise × V-2), 95P63-11 (Spunta × V-2), 95P87-4 (Frisia × V-2), 96YS51-1 (Atlantic × BR63-66), 96YS21-13 (LT-8 × Tobique), 97YS211-3 (Hertha × V-2), 97YS215-3 (LT-9 × Vanesa), 99YS181-10 (Bartina × Bulk), 99YS185-1 (Cinja × Bulk), 99YS188-3 (Hertha × Bulk), 99YS191-1 and 99YS191-8 (LT-8 × Hermes), and 99YS192-5, 99YS192-9 and 99YS192-12 (Maris Piper × Bulk); Rysto genotypes: Bzura, Forelle, Pirola, White Lady and breeding clones 94APP-2 (Forelle × Sandra) and 94APP-4 (Pirola × Leila); varieties resistant to *G. rostochiensis* with the Gro1-4 gene Optima, Valetta and 94APP-3 (Optima × Valetta), and with H1 Atlantic, Cara, Lady Claire, Santé and Saturna; and finally, varieties with resistance to *G. pallida* Pa2,3 Santé and Innovator. These varieties had been obtained in previous years from different breeding institutions or gene banks and maintained in the progenitor collection of Appacale.

4.1.2 Traceability

A total of 144 samples consisting of a batch of 15-20 fresh tubers (3-5 kg) were collected from 2003 to 2011 by clients in different locations and from different origins (supermarkets, distributors.). All the samples were under suspicion of fraud or mislabeling. To evaluate the impact of this potential fraud in the Spanish market, other 18 samples consisting of a batch of 15-20 fresh tubers (3-5 kg) were collected in 2011 in seven of the top supermarket chains in Spain. The samples were collected at random in supermarkets from two different locations in northern Spain. In all cases, 3 tubers were chosen at random for DNA analysis. The number of samples of each potato variety sampled ranged between 1 (Lady Christl, Blanka and Asterix) and 77 (Monalisa) in the samples collected from 2003 to 2011, and between 1 (Axterix, Elodie) and 8 (Monalisa) in the samples collected at random in 2011 42% of the samples collected

between 2003 and 2011 had national origin, while a 28% had foreign origin, mainly France (90% of the foreign samples) (Fig. 3.1). Origin of the rest of the samples was unknown. Almost 28% of the samples collected at random in 2011 had a national origin. The rest were all from foreign origin (France) (Fig. 3.2).

4.1.3 Transgenics

A total of 13 samples consisting of a batch of 15-20 fresh tubers (3-5 kg) were collected in 2014 in five of the top supermarket chains in Spain (Fig.3.3). The samples were collected at random in supermarkets from two different locations in northern Spain. In all cases, 3 tubers were chosen at random for DNA analysis. The number of samples of each potato variety sampled ranged between 1 (Soprano, Vivaldi, Laura, Mozart, Elodie, Rudolph and Orquesta) and 4 (Agata). 69% of the samples collected had national origin, while a 31% had foreign origin, mainly France (75% of the foreign samples).

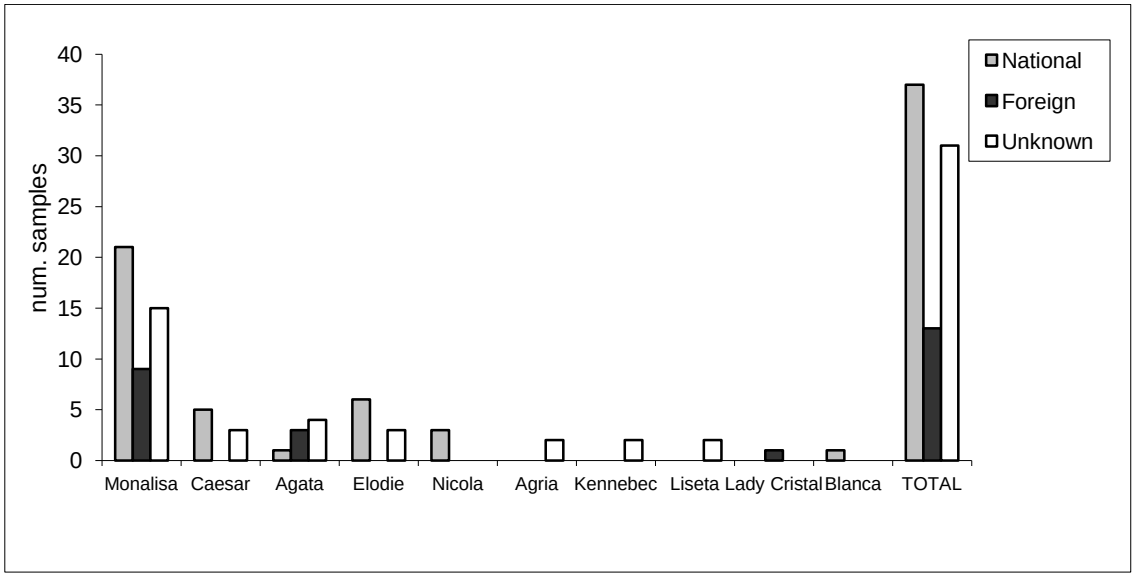

Fig.3.1. Origin of the mislabelled samples for each variety and in total. in samples collected under suspicion of mislabelling (2003-2011).

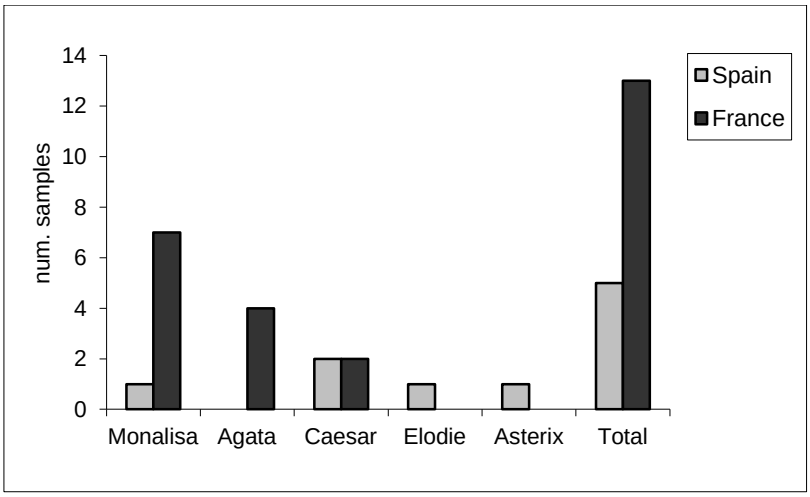

Fig.3.2. Origin of the mislabelled samples for each variety and in total. in samples collected at random (2011).

Fig.3.3. Samples collected at supermarkets chains in Spain.

4.2 Genetic Analysis

4.2.1 Molecular Markers

4.2.1.1 Breeding

The Ryadg and Rysto genes for resistance to PVY were identified using the SCAR marker RYSC3 described by Kasai et al. (Kasai *et al.*, 2000) and marker STM0003 described by Song et al.

(Song *et al.*, 2005), respectively (Table 1). The Gro1-4 locus of resistance to *G. rostochiensis* was selected by using specific primers for the Gro1-4 resistance gene adapted from Gebhardt et al. (Gebhardt *et al.*, 2006). The TG689 protocol to select the H1 gene of resistance to *G. rostochiensis* was kindly provided by W.S. De Jong. Thus, PCR was performed in a total volume of 20 µl containing 0.4 µl 10 µM of TG689 allele specific and TG689 indel12 primers, 0.2 µl of primers DCH-F2 and 10 µM DCH-R2, 2 µl 10× PCR buffer, 1.6 µl 2.5 mM dNTPs, 2 µl DNA and 0.2 µl 5U/µl DFS-Taq DNA polymerase (Bioron GmbH). PCR conditions were: initial denaturation for 2 min at 94 °C, 35 cycles of 20 s at 94 °C, 30 s at 55 °C and 3 min at 72 °C, and one cycle of final extension for 5 min at 72 °C. PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. SPUD1636 for *G. pallida* resistance was implemented from Bryan et al. (Bryan *et al.*, 2002) and the HC marker assay is described by Sattarzadeh et al. (Sattarzadeh *et al.*, 2006). Markers were applied on the second year of field trials of the breeding clones.

4.2.1.2 Traceability

SSRs were selected from previous studies that identified SSRs showing high levels of polymorphism in tetraploid potato (Provan *et al.*, 1996; Milbourne *et al.*, 1998). The two primer pairs used in this study are listed in Table 3.1.

Locus	Motif repeat		Primer sequence (5′–3′)	Tm (°C)
STM2020[a]	$(TAA)_6$	F	CCTTCCCCTTAAATACAATAACCC	60.2
		R	CATGGAGAAGTGAAAACGTCTG	59.8
STIIKA[b]	$(T)_{12}$ $(A)_9$ ATTCTTGTT	F	TTCGTTGCTTTACCTACTA	50
	$(TA)_2$ CA $(TA)_7$	R	CCCAAGATTACCACATTC	50

Primer/locis, sequence and repeated motif acquired from published literature.
[a] Milbourne et al. (1998).
[b] Provan et al. (1996).

Table 3.1. Primers pairs used in the study.

4.2.1.3 Transgenics

Biogenics Standard Kit for GMOs detection (Biotools B&M Labs.S.A., Spain) was used for the detection and amplification of GMOs specific regions not present in native plants (35s promoter and NOS terminator). These regions are present in approximately 90% of marketed plant GMOs to date.

4.2.2 DNA isolation

DNA was extracted from the skin of the tuber in the case of the collected samples according to (Edwards K, Johnstone C, 1991). DNA extraction from stated and control varieties was performed using tuber skin of leave tissue, according to the same protocol. 6mg of fresh tissue were ground and collected in 400 ml of extraction buffer (0.2 M Tris (pH 7.5); 0.25 M NaCl; 0.025 M EDTA; 0.5% SDS). The tube was shaken in vortex and incubated at room temperature for 30 min. After centrifuging for 1 min at 13000 rpm, the supernatant was transferred to a new tube. 320 ml of isopropanol were added for precipitation and after centrifuging for 5 min at 13000 rpm and drying, the pellet was recovered in 400 ml of TE (10 mM TriseHCl, pH 7.4; 1 mM EDTA, pH 8). All DNA extractions were repeated twice.

4.2.3 PCR conditions

4.2.3.1 Breeding

The conditions for PCR amplification of the SCAR marker RYSC3 were as described by Kasai et al. (Kasai *et al.*, 2000) and as described by Song et al. (Song *et al.*, 2005) for marker STM0003 respectively (Table 1). The PCR conditions for the amplification of the specific primers for the Gro1-4 resistance gene adapted from Gebhardt et al. (Gebhardt *et al.*, 2006) were as described by the authors. The TG689 protocol to select the H1 gene of resistance to *G. rostochiensis* was kindly provided by W.S. De Jong. Thus, PCR was performed in a total volume of 20 µl containing 0.4 µl 10 µM of TG689 allele specific and TG689 indel12 primers, 0.2 µl of primers DCH-F2 and 10 µM DCH-R2, 2 µl 10× PCR buffer, 1.6 µl 2.5 mM dNTPs, 2 µl DNA and 0.2 µl 5U/µl DFS-Taq DNA polymerase (Bioron GmbH). PCR conditions were: initial denaturation for 2 min at 94 °C, 35 cycles of 20 s at 94 °C, 30 s at 55 °C and 3 min at 72 °C, and one cycle of final extension for 5 min at 72 °C.

4.2.3.2 Traceability

PCR was carried out in a total reaction volume of 10 ml containing 10X PCR buffer [500 mM KCl, 100 mM TriseHCl pH 8.3, 15 mM Mg(OAc)2], 200 mM dNTPs, 0.5 mM each primer, 0.5 U Taq DNA polymerase (Bioron GmbH, Ludwigshafen, Germany) and 2 ml of DNA template DNA. All amplifications were carried out in an Applied Biosystems (Foster City, CA, USA) 9700 thermo cycler using the following parameters: 94°C for 1 min; 94° C for 30 s, [Tm] for 30 s, 72° C for 1 min _ 44 cycles; 72° C for 15 min, (for Tm values refer to Table 3.1).

4.2.3.3 Transgenics

PCR was carried out following Biogenics Kit protocol, modified as follows: PCR was performed in a total reaction volume of 25 µl containing 1.25 µl MgCl2, 7.5 µl Master Mix, 0.7 µl Taq, 10.55 µl H2O and 5 µl DNA. All amplifications were carried out in an Applied Biosystems (Foster City, CA, USA) 9700 thermo cycler using the following parameters: 94° C for 3 min; 94° C for 30 s, 55° C for 40 s, 72° C for 1 min _ 45 cycles; 72° C for 3 min.

4.2.4 Genotypes visualization

4.2.4.1 Breeding

PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining under UV light.

4.2.4.2 Traceability

PCR products were denatured by the addition of 10 ml stop solution (95% formamide) and heating to 94 _C for 5 min. Then 3 ml of each sample were loaded onto 6% polyacrilamide denaturing gels (8 M urea) buffered with 1X TBE and separated for 1-2 h at 90 W constant power using a DNA sequencing gel electrophoresis apparatus (BioRad, Hercules, CA, USA). Gels were stained with the method of Bassam *et al*. (Bassam *et al.*, 1991) modified as follows: gels were fixed for 45 min in 10% acetic acid; washed with distilled water for 5 min (2X); stained for 45 min in freshly prepared 0.1% AgNO3, with 1.5 ml of formaldehyde; rinsed for 4e5 s with distilled water; developed for about 5 min in freshly prepared 3% Na2CO3 with 1.5 ml of formaldehyde added immediately prior to use; and fixed for 5 min in 10% acetic acid. The

genotype of each sample was compared with the genotype of stated and control varieties by amplifying them in the same PCR and then running them in the same gel. PCR was performed twice for each sample. The 3 tubers of each sample were analysed separately. Stated and control varieties were obtained from a Potato Gene Bank (NEIKER Potato Germplasm Bank, Spain) or from the correspondent Breeder (C. Meijer CV, HZPC Holland B.V.), which assures their authenticity.

4.2.5 Transgenics

PCR products were separated by 1% agarose gel electrophoresis at NN V for approximately 15 min, and visualized with ethidium bromide staining under UV light.

4.3 Statistical analysis

4.3.1 Calculation of segregation ratios

The total analyses performed in the progenies descending from the same parent (variety or breeding clone) used as resistance donor were pooled, and the segregation ratios of the markers in those progenies compared with the expected ratios of chromosome (simplex, 1:1 resistant/susceptible; duplex, 5:1; triplex, quadruplex 1:0) and chromatid segregation (simplex, 0.86:1 resistant/susceptible; duplex, 3.67:1; triplex, 27:1; quadruplex, 1:0) with a chi-square goodness-of-fit test with the Yates correction for continuity, according to Zar (Zar, 2010). Only progenies with more than 100 tests per parent were included in the analysis.

4.3.2 Estimates of improvement costs

The cost of applying any of the mentioned markers to one genotype was estimated, considering that about 30 assays are usually performed at the same time. Thus, total labor time for DNA extraction, PCR and electrophoresis was estimated and then divided by 30 to know how many minutes corresponded to each genotype, and then translated into euros according to the cost of 1 h of work of the personnel involved. Costs of primers, Taq polymerase and agarose or acrylamide were specifically calculated for each primer pair. A

common amount of 0.72 € for fungible material as tubes, tips, buffers, etc. was added to all of them. Indirect costs were not considered.

4.3.2.1 Validation of STM0003 Marker

To test if the marker STM0003 identified the PVY resistance, all the breeding clones that were positive for the STM0003 marker and were selected in their second year of field trials because of their good agronomic performance (the same year that molecular markers are applied, see above) were tested the following year (third year of field trials) inoculations were estimated for the standard procedure that is usually used at the company (inoculation tests were not performed). According to this protocol, three virus-free tubers per genotype are grown in 10-cm pots in a greenhouse at 15 °C (range, 15–30 °C) with natural long-day summer light (April–September). Once grown (about 6 weeks later), plants are infected with sap from tobacco fresh leaves checked for infection with PVYo or PVYNTN. Symptoms are recorded and all plants tested at least once by enzyme-linked immunosorbent assay (ELISA) about 4 weeks after inoculation. Plants that exhibit visual symptoms or/and give a positive ELISA result would be considered as susceptible and symptomless response and negative ELISA results as resistant. Inoculations are repeated the second year in all genotypes considered as resistant to confirm their resistance. Labour costs of planting, watering, inoculation, ELISA tests, etc. per genotype were calculated, plus material costs like pot soil and ELISA antiserums. Indirect costs were not included.

4.3.2.2 Cyst Nematode Artificial Inoculation and Cost Estimation

Artificial inoculations to test nematode resistance were developed following the procedure described by the Council Directive 2007/33/EC. Thus, the tests were performed in climate chambers under controlled conditions of light and humidity. Variety Désirée was used as susceptible standard and *G. rostochiensis* population Ecosse as inoculum to test Ro1 resistance. The inoculum consisted of a total of five infective eggs per milliliter of soil. We used six replicates per variety. Plants were cultivated during 3 months. Cysts from the six replicates were extracted with a Fenwick can and counted separately for each pot. The relative susceptibility of the tested varieties or breeding clones was expressed as a percentage related

to the final population of the susceptible control and scored from 9 (resistant) to 1 (susceptible). Labour costs of preparing the inoculums, planting, watering, washing sand, counting cysts, etc. per genotype were calculated. Indirect costs were not included.

4.3.3 Comparison for samples with fraud level.

Comparison of levels of mislabeling between samples collected under suspicion of fraud and samples collected at random was analyzed using Yates correction for continuity.

5. RESULTS

5 RESULTS

5.1 Resistance markers

From samples obtained since 2000, 1,940 tests were performed on the progenies that could amplify markers RYSC3, STM0003, Gro1-4, TG689, SPUD1636 and HC based on their pedigree; 778 (40.1%) of them amplified any of the markers and were regarded as resistant (Table 4.1). Some of them are still maintained as advanced breeding material or are actually being tested in the official registration assays to be released as new varieties. Among them, 1,197 genotypes were tested with RYSC3 marker, 451 (37.7%) of them being positive for the marker and 140 (11.7%) selected in their second year of field trials for further selection (Table 2). Seven of these clones are still maintained at the breeding program as breeding material. Besides, in samples since 2006, 536 assays have been performed with STM0003 marker to select PVY resistance conferred by Rysto gene, 233 (43.5%) of them being positive and thus regarded as resistant (Table 3). Fifty nine of these clones (11.0%) were selected because of their good agronomic performance, and seven of them are still in the breeding programme. However, adding up the results of 2006 to 2010, we found that 5 of these 59 breeding clones that amplified STM0003 were positive for PVY infection the year after the marker test was performed. The presence of Gro1-4 locus of *G. rostochiensis* Ro1 resistance was evaluated in 43 breeding clones, 15 of them being positive (34.9%; Table 4). Seven of them (16.3%) were selected because of their good agronomic performance, and two of them are advanced material. On the other hand, varieties that could have H1 gene and six breeding clones were checked for the presence of the RFLP marker TG689 in 2010, with all the varieties and two of the breeding clones being positive (33.3%; Table 1). Also, 16 breeding clones analysed with markers Gro1-4 and TG689 were inoculated with *G. rostochiensis* Ro1 cysts (Table 5). All the resistant clones amplified markers Gro1-4 or TG689 according to their pedigree, and susceptible ones did not. Finally, 158 genotypes were tested with markers SPUD1636 or HC for *G. pallida* Pa2,3 resistance. Seventy seven of them had the QTL of resistance (48.7%; Table 4.1).

Pathogen	Gene	Chr	Marker	Reference	Years	Total analysed	Positive
PVY, PVA	Ry_{adg}, N_{adg}	XI	RYSC3	Kasai et al. (2000)	2000–2010	1,197	451
PVY	*Rysto*	XII	STM0003	Song et al. (2005)	2006–2010	536	233
G. rostochiensis	*Gro1-4*	VII	Gro1-4	Gebhardt et al. (2006)	2008–2010	43	15
G. rostochiensis	*H1*	V	TG689	W.S. De Jong (personal communication)	2010	6	2
G. pallida	*RGpa5*	V	SPUD1636	Bryan et al. (2002)	2010	6	1
G. pallida	*RGpa5-vrnHC*	V	HC	Sattarzadeh et al. (2006)	2007–2010	152	76
Total						1,940	778

Table 4.1. Molecular markers applied in the company and total number of molecular marker analyses performed in the breeding clones of the company's programme since 2000 to select disease resistance

5.1.1 Economical costs

Regarding the economical costs of MAS, the costs of phenotyping with artificial inoculations for PVY and cyst nematodes were compared with the costs of applying each marker (Table 6). As it can be seen, molecular markers were less expensive if the resistance could be selected with one or two diagnostic markers, with costs reduced in 76%-96% compared to those needed for artificial inoculations (PVY and PCN respectively).

5.1.2 Segregation ratios

In addition, the segregation ratios of the markers in those progenies with more than 100 genotypes analysed per parent were compared with the expected ratios of chromosome and chromatid segregation. Results are shown in Table 7. In four of the six parents analysed (66.7%), there were no significant differences between the ratios observed and expected for the simplex dosage, either with chromosome or chromatid segregation. However, in two cases, the progenies did not fit any expected ratio.

5.2 Traceability

The two primer set employed in this work, allowed to distinguish between the eleven different varieties of potato analyzed, establishing a unique pattern for each variety (Fig. 4.1).

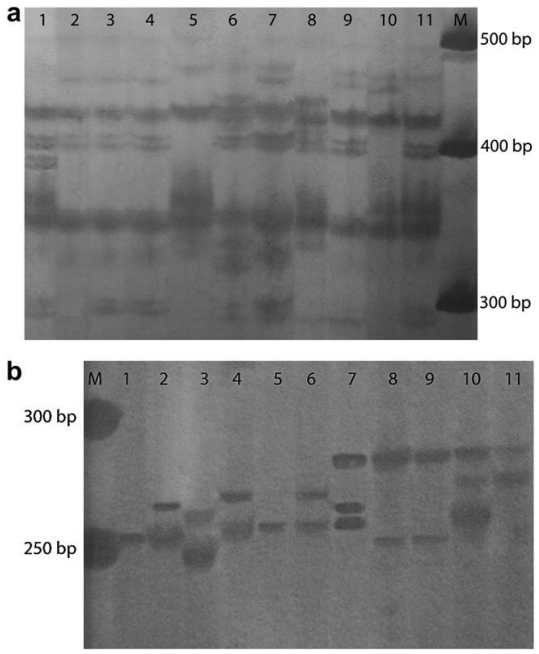

Fig.4.1. SSR profiles of the *S. tuberosum* cultivar controls. a) for loci STIIKA (Provan et al. 1996) 1e11: 1, Lady Christl; 2, Kennebec; 3, Caesar; 4, Monalisa; 5, Elodie; 6, Asterix; 7, Agata; 8, Nicola; 9, Liseta; 10, Blanka; 11, Agria; M, Molecular weight ladder in base pairs (bp). b) for loci STM2020 (Milbourne et al. 1998) 1e11: 1, Caesar; 2, Elodie; 3, Asterix; 4, Agria; 5, Liseta; 6, Kennebec; 7, Lady Christl; 8, Monalisa; 9, Blanka; 10, Agata; 11, Nicola, M, Molecular Weight Ladder in base pairs (bp).

5.2.1 Samples collected under suspicion of fraud

High proportion of the total samples were amplified successfully (about 99%) with both pairs of primers and compared with the authenticated references In the samples from 2003 to 2011, (under suspicion of fraud or mislabeling) 10 different varieties were analyzed with different number of samples: from 1 (Lady Christl and Blanka) to 77 (Monalisa). The number of mislabeled samples ranged between 0 (Lady Christl and Agria) and 45 (Monalisa) (Fig. 4.1). Total number of mislabeled samples was 78 out of 144 (54.2%). In the 98.4% of the cases, mislabeling was established for the 3 tubers analyzed in each sample. In the remaining 1.6% (1 case) mislabeling was established for 2 of the 3 tubers analyzed in the sample. A 6.2% of the

mislabeled samples showed more than one genotype not corresponding with the labeled variety. In total 37 of the mislabeled samples (45.7%) were from national origin, while 13 (16.0%) had foreign origin.

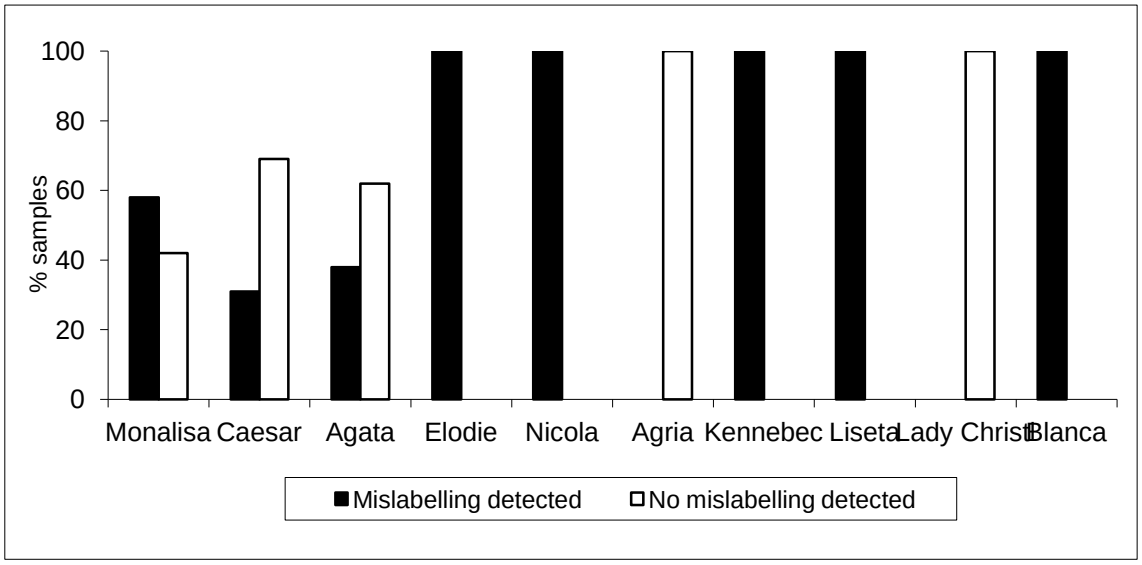

Fig.4.2. Number of samples showing mislabelling for each variety in samples collected under suspicion of mislabelling (2003-2011).

5.2.2 Samples collected at random

High proportion of the total samples were amplified successfully (about 99%) with both pairs of primers and compared with the authenticated references. In the samples collected at random in different supermarkets in 2011 (Table 1. B.), 5 different varieties were analyzed with different number of samples: from 1 (Elodie and Asterix) to 8 (Monalisa). Number of mislabeled samples ranged between 0 (Asterix and Caesar) and 2 (Monalisa) (Fig. 3. b). Total number of mislabeled samples was 4 out of 18 (22.22%). In the 75% of the cases of mislabeling, it was established for the 3 tubers analyzed in each sample. In the remaining 25% (1 case) mislabeling was established for 2 of the 3 tubers analyzed in the sample. A 25% of the mislabeled samples showed more than one genotype not corresponding with the labeled variety. 1 of the mislabeled samples (25.0%) was from national origin, while 3 (75.0%) had foreign origin (France) (Fig. 4.2).

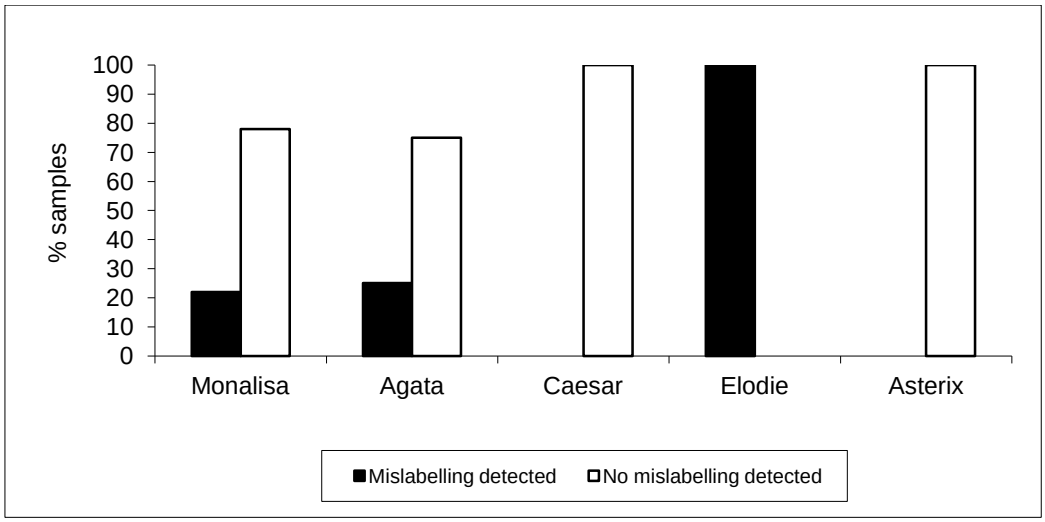

Fig.4.3. Number of samples showing mislabelling for each variety in samples collected at random (2011).

5.2.3 Comparison between samples collected under suspicion of fraud and samples collected at random

The differences found between the mislabeling in the samples that were under suspicion of fraud and the mislabeling in those that were sampled at random were highly significant (Yates χ^2=0.0, p-value ¼ 1), being higher the mislabeling detected in the samples collected under suspicion of fraud than in those collected at random.

6. DISCUSSION

6 DISCUSSION

In this work I have demonstrated the usefulness of microsatellite loci for application in improvement and traceability of potato cultivars. This represents a noticeable advance for this organism. Molecular markers are being widely applied to assist many breeding programs worldwide; as well as tools for food traceability. However, while in crops like wheat, rice or tomato, they have became common tools and a lot of research has been done in both molecular marker-assisted breeding (Visscher *et al.*, 1996; Dubcovsky, 2004; Semagn *et al.*, 2006; Gupta *et al.*, 2009; Singh *et al.*, 2011) and traceability (Vietina *et al.*, 2011; Martins-Lopes *et al.*, 2013; Sardaro *et al.*, 2013), the potato crop remains a little more traditional than other crops, that are less important when it refers to world production.

6.1 Molecular Marker-Assisted Breeding

Information about the discovery, mapping, validation and practical application of the qualitative and quantitative genes conferring resistance to important diseases and pests affecting potato, in the form of MAS in practical breeding programmes, has not been very common so far. RYSC3 SCAR marker was the first applied and validated by a private company as reported by Arcaute R., Isla S., Carrasco, (2002) in Appacale's Breeding Programme; afterwards, no more phenotyping and validation work have been done. Also, more recently, it has been reported the validation and implementation of this marker in the US Pacific Northwest Potato Breeding Programme (Ottoman *et al.*, 2009). PVY resistance from the wild species *Solanum stoloniferum* (Rysto) has been selected with STM0003 marker (Song *et al.*, 2005). We found that the 8.3% of the selected clones regarded as resistant (because they amplified the marker), were infected with PVY, according to ELISA test. This result may be due to errors in ELISA or PCR assays or to recombination. In any case, the marker was able to identify effectively the resistance in almost 92% cases, which we consider justifies its use as a tool to select the PVY resistance in the breeding programme.

Regarding PCN resistance, and according to the artificial inoculations performed, Gro1-4 and TG689 were suitable for the breeding programme since all the clones tested phenotypically showed levels of resistance according to their genotypes. However, although no

recombination event was observed, the number of genotypes tested was too small to reliably assess the frequency of false positives due to recombination. Gebhardt *et al.*, (2006) also found a total correspondence among Gro1-4 amplifying genotypes and their phenotypes, supporting the suitability of this marker as a diagnostic tool.

On the other hand, the diagnostic value for DNA markers SPUD1636 and HC linked to *G. pallida* resistance was demonstrated (Bryan *et al.*, 2002; Sattarzadeh *et al.*, 2006) respectively. Both markers have been used for *G. pallida* resistance selection. However, no phenotyping work has been performed to validate this marker. Reasons are, inoculation tests for *G. pallida* are often ambiguous, and also because, in this case, the markers are diagnostic for a QTL, not a major gene. I've trusted the information provided by Bryan *et al.*, (2002) and Sattarzadeh *et al.*, (2006), even if the results were not 100% effective. The deployment of effective resistance against *G. pallida* is difficult because of its genetic complexity. In this situation, the availability of two markers that identify a great amount of the genetic resistance is highly valuable for the breeding programme, even if no complete resistance is achieved.

An additional advantage of molecular markers is the possibility to know the allele dosage of the resistance gene of each parent based on the segregation ratios of the markers in the progenies. All the parents analysed have the gene of resistance in simplex, but some of them could have had it in duplex or even triplex. This is very interesting because it is possible to construct superior parents with multiple copies and thus obtain full-sib resistance progeny. Currently, the PCR assays used are not dosage sensitive; therefore we depend on progeny testing to determine the number of resistant alleles present in a particular parent. But even so, progeny testing is simpler, quicker and cheaper with molecular markers than with artificial inoculations. Recently, a specific study to know the allele dosage of some of the parents used in the company as donors of resistance to *G. rostochiensis* linked to Gro1-4 marker has been performed, identifying one parent with the gene in duplex dosage (López-Vizcon and Ortega, 2011). In the other cases evaluated, the progenies did not fit any expected ratio. This could be due to errors in the marker assays or more probably in the crosses performed to obtain the breeding clones. For example, in the case of the breeding clone 95APP-5, when it was used as female, chi-square values fit a simplex dosage ($\chi2_{chromosome}$=3.11, $\chi2_{chromatid}$=1.72, N=63), but when it was used as pollinator, the dosage was not clear (χ2 chromosome 0 5.44*, χ2 chromatid 0 3.04, N089). This could indicate undesired pollinations or errors in the classification of berries in some of the crossings performed to obtained seeds with 95APP-5 as pollinator.

The breeding programme carried out in the host company Appacale follows the traditional scheme of crossings, seedlings, first year of field trials in a one-tuber plot per genotype at seed site, second year of field trials in a seven-tuber plot at seed site, etc. In this system, there is a huge selection pressure in the first years of clonal selection in the field based only on general appearance and agronomic performance, giving a reduction of about 85–90% of the starting material. PVY and *Globodera* spp. markers are applied in the second year of field trials, after the first year sieve, because the breeding process is a permanent counterbalance between costs and benefits; applying the markers in the first year, when most of the material will be discarded, would be too expensive and not efficient at all. Moreover, breeding clones analysed with molecular markers are selected based on their phenotype plus their genotype. That means that being positive for the marker is considered as an important plus, but not all of the positives are selected and not all the negatives are discarded. All the evaluated genotypes have to accomplish a minimum of agronomic performance to pass to the following year of selection. This is an important point because there are some varieties that produce progenies that can be selected with molecular markers but produce very unfit ("bad") descendants. This is an important limit for MAS, as will be discussed later. MAS in the seedling generation is too costly due to the large number of DNA extractions and PCR assays required. For example, in 2010, 26,184 seeds were sown at the seedlings step, and 14,833 clones were planted in the first year of field trials; 9,021 and 8,118 of them, respectively, could have amplified any of the markers. Assuming that about 90% of the seeds were able to germinate and there were losses due to sprouting problems or others in the field, about 7,300 or 8,000 analyses should have been done, respectively. On the contrary, 225 marker analyses were performed in the second year breeding clones. Translated into money, multiplying each analysis per a mean amount of 3€ (see Table 6), the costs vary from 21,900€ at the seedlings step or 24,000€ in the first year of field trials to 675€ in the second year of field trials. Thus, although it has been traditionally said that molecular markers are too expensive to be systematically used, if they are applied in the suitable step of a breeding programme (that should be decided by each company or institution), it is obvious that molecular markers allow an early selection of resistant clones in an affordable way.

Comparing the costs of molecular markers and artificial inoculations, markers provide an earlier, quicker, cheaper and more reliable system of screening for the resistances. This is true only for published markers; developing new ones is not affordable nowadays for a small breeding company, like Appacale where the selection experiments of this Thesis were carried

out. Added to other well-known benefits of MAS, another advantage of special interest for breeding is the possibility to pyramidize genes in one genotype as demonstrated by Gebhardt *et al.*, (2006)). At this moment, the host company has four breeding clones with more than one resistance that are being used as parents. The progress was not as fast as reported by Gebhardt *et al.* (2006) firstly because, until 2006, only RYSC3 marker could be applied at the company and, secondly, because at least one year of field selection is performed before applying the marker. Thus, the minimum time needed until a genotype is used as a parent is 4 years (crossings, seedling, 2 years of field trials). An additional problem for practical breeding is that not all the genotypes are able to flower, so they have no use as parents even if they have multiple resistances. In other cases, the resistance is linked to male sterility, as in the case of Rysto resistance (Ross, 1986), and they can only be used as female parents, limiting their use.

So far, it has been shown that the benefits and usefulness of MAS to select interesting resistances are many. Despite this, MAS has been only of limited relevance in commercial potato breeding programmes due to different handicaps, some of them reported above. Probably, the most important of them is that markers can only be applied to certain genetic background, and therefore, there are still large amounts of resistances that cannot be selected with this procedure. For instance, nowadays in Appacale there are 32 highly PVY-resistant parents in the progenitor collection, according to their personal communications and also the European Cultivated Potato Database (www.europotato.org), but only six (19%) and nine (28%) of them have the Ryadg or Rysto genes for their progenies could be selected with RYSC3 or STM0003 markers, respectively. The same holds for *G. rostochiensis*: there are 50 parents resistant to pathotype Ro1-4 in the company's gene bank, but only 11 of them amplify Gro1-4 (22%) and 5 TG689 (10%). In the case of *G. pallida*, the sources of resistance are really scarce; there are only four resistant varieties, and three of them could be selected with SPUD1636 or HC markers. Additionally, some of the varieties that can be selected with markers produce progenies that could be resistant but have very low agronomic value. This is because, by reason of feasibility, linkage analysis of quantitative and qualitative agronomic traits in potato using DNA-based markers is mostly being performed in experimental, diploid or interspecific mapping populations, not well adapted and with fewer general agronomic qualities than advanced breeding clones or cultivars. This is shown by the Ryadg selection rates in Table 3.2. In that table it can be seen that the level of selection of clones obtained from Ryadg varieties is near or slightly superior to the mean value of the general breeding programme for that year of field trials, which varies from 17% to 25% of the clones (data not shown). However, none of these breeding clones "has gone further" than the third year of field trials; they have not been

successful at all. Ryadg varieties 7XY-1, LT-8, LT-9, P-6 or V-2 were bred by the International Potato Centre (Lima, Peru), and they are not well adapted to European long-day conditions, so they usually produce progenies with dormancy problems, among others, that make them not suitable for European conditions. This tendency is reduced to some extent when the first backcrosses of these varieties are used as resistance donors (99YS191- 1, 99YS191-12, etc.), but even so, their success is low. In the case of Rysto, it is not possible to compare among varieties because there are few data out from the Hungarian variety White Lady; however, the progenies of this variety seem to have a good field performance, much better than Ryadg varieties.

In conclusion, the application of MAS is severely limited by the low number of varieties that could amplify the markers, the capability of these varieties to flower and produce seeds and the agronomic performance of the progenies obtained. Another reason for the limited number of published reports about the practical use of MAS could be that private seed companies usually do not give details of their methodology due to competition with other companies. In general, the problem of publishing extends also to the scientific community. New QTLs or R genes are often reported in scientific journals, but reconfirmation of these QTLs and R genes in other germplasm and identification of more useful markers are usually not considered novel enough to justify new publication. This is exactly the information needed for MAS application. Finally, the low impact of MAS could also be due to the fact that although DNA markers were first developed in the late 1980s, user friendly ones such as SSRs were not developed until the late 1990s. With the advent of third-generation marker technologies such as the single-nucleotide polymorphisms that can be genotyped using Next Generation Sequencing, the power and efficiency of genotyping are expected to improve in the coming decades, and the promise of MAS for improving polygenic traits in a quick time-frame and a cost-effective manner could be a reality. However, it is also worth noting that large initial capital investments are required for the acquisition of this third-generation equipment, maintenance or personnel training and that this can widen even more the gap between the scientific and breeding communities or among well-developed and developing countries, reinforced by the secrecy or protection of intellectual property rights. Any SME or breeding institution should have to compare the costs of implementing the equipment required and the benefits of applying these technologies along the time. Moreover, to take advantage of these technologies, research teams, governmental agencies, commodity groups and the commercial sector will need to work together to ensure a tangible impact on crop improvement.

6.2 Traceability

In this study a 22.2% of mislabelling was detected for the 18 samples collected at random, which differs (Yates c2 ¼ 0.0, p-value ¼ 1) to the percentage detected on the under suspicion of fraud samples (54.2%). That indicates that mislabelling in the Spanish potato market does exist, although it is not as high as the first studies using under suspicion samples showed (REF.???). The difference in the mislabelling percentage may be not only because the first samples were collected under suspicion of fraud and the second ones were collected at random. It could also be due to the origin of the samples. The samples under suspicion of fraud were collected in supermarkets but also from distributors or independent/small chains, while the samples collected at random were all from supermarkets. It is expected that the mislabelling from these different sources can be very different as well.

The results also showed that this mislabelling is recurrent in different years and not just a punctual problem. The mislabelling levels detected in the potato market are a bit lower than the levels found in other Spanish food products, like it is shown in a recent study about mislabelling in hake, where 38.9% of mislabelling is detected (Garcia-Vazquez *et al.*, 2011); but still, the levels are significant. Moreover, the employment of a larger primer set could maybe allow detecting higher levels of mislabelling. Although many studies describing traceability methodologies for the Spanish food industry have been developed (e.g. Fajardo *et al.*, 2006; Fernández-Tajes *et al.*, 2010; Rojas, M. *et al.*, 2010), not many scientific studies have been focused on the evaluation of real mislabelling and fraud levels in the Spanish market. These kinds of studies provide useful and necessary information to choose the best strategies to avoid and prevent mislabelling. Under current EU legislation, there is a requirement for potatoes that are offered for wholesale or retail-sale to be labelled with their variety name (EU Directive 2003/89/EC). Consumers have the right to know what they are buying and consuming. Most potato varieties are classified and prized depending on their characteristics and end-use. Consumers may purchase a particular variety of potatoes for any of the above reasons. Thus, this fraudulent substitution is unfair for the consumers, because they pay for a product that they are not buying in reality.

The study presented in this Thesis was focused exclusively on the Spanish market, but the origin of the samples that were analysed was not only national; in total, 66% of the samples came from foreign origins, mainly France (92% of the samples with foreign origin). Moreover,

on average, a 45% of the mislabelled samples were from foreign origin. This could suggest that the same problem could be occurring in other countries as well. In 2003, the Food Standards Agency of the United Kingdom ran a similar study (www.food.gov.uk/multimedia/pdfs/fsis4403.pdf) with revealing results. They found a high percentage (33%) of samples without appropriate labelling. However, this percentage was much lower (2%) when analyzing samples coming only from big supermarkets, while it was found that small stores or chains, market stalls and wholesale markets were responsible for most of the mislabelled samples, which is consistent with my results. The study also confirmed that mislabelling in potato is a problem not only in Spain but in other European countries. The study of the Food Standards Agency of the United Kingdom not only showed a high mislabelling percentage in the British potato market, they also found a problem with one of the most appreciated varieties in UK (King Edward), with a high percentage of mislabelling in the wholesale or market stalls. I believe that something similar could be occurring in Spain with variety Monalisa, one of the most appreciated and demanded varieties in Spain (MAGRAMA, 2011). Most of the samples collected under suspicion of fraud belonged to this variety, and a high percentage of mislabelling was found for this cultivar in the samples that were under suspicion of fraud and also in those collected at random.

It is important to point out that, by the time that the samples were collected at random (January-February), imported potatoes are stored potatoes coming mainly from France. Taking into account the results already obtained, it would be interesting to develop a similar study along May and June, when early potatoes start arriving from southern Spain, Morocco or Israel. That could let us evaluate whether or not does mislabelling exist in potatoes coming from these countries and if so, we could compare the results with the ones obtained in this work. It would also give us a wider perspective about mislabelling in the potato market not only in Spain or the EU, but in other important world potato producers. This study was carried out using SSRs as an effective and reliable genetic tool for cultivar identification. Molecular markers have proved to be a helpful, affordable and easy-to-use tool for food traceability; this should encourage administrations to increase routine controls in order to avoid fraud and ensure consumers rights. From my results and from other studies in different products and markets, it is evident that more efforts should be done in traceability and food control in Spain and in the rest of the EU, and genetic tools give the possibility to enforce all these actions.

6.3 Insights in potato transgene traceability

The last part of this Thesis has been devoted to test the possible presence of transgenes using molecular markers. Results on mislabelling fraud in Spain and other EU countries, and the low relevance of molecular tools applied to food control in the potato industry, together with the raising concern of consumers about food quality and safety and GM food (Maghari and Ardekani, 2011; Galimberti *et al.*, 2013; Hall, 2014) point out the need to start developing and testing reliable tools for GMOs traceability and/or fraud in the potato market as well.

Polymerase chain reaction (PCR) is the method of choice used by most analytical laboratories for the detection of GMOs and GMO-derived materials because of its high sensitivity, specificity and its capability to detect wide range of constructs ((Gachet *et al.*, 1998); Holst-Jensen *et al*., 2003; James *et al*., 2003; Forte *et al*., 2005; Hernandez *et al*., 2005). In addition, a confirmatory assay of the identity of the amplicon is required to ensure that the amplified DNA product actually corresponds to the chosen target sequence and is not non-specific binding of primers during PCR amplification (Wolf *et al*., 2000; Anklam *et al*., 2002). DNA sequencing is the most reliable method to confirm the authenticity of PCR products (Gachet *et al*., 1999; Anklam *et al*., 2002). As explained in the Introduction, the NOS gene is located in the transferred DNA (T-DNA) of the Ti Plasmid in *Agrobacterium tumefaciens*. The gene is transferred along with the T-DNA to plant chromosomes following *Agrobacterium*-mediated plant transformation and is expressed in many plant cells.

The results in this study, indicate the potential presence of NOS terminator, a GMOs specific region not present in native plants and present in approximately 90% of marketed GMOs to date in 53.8 % of the 13 samples collected at random and analysed. None of the samples amplified successfully for the primers pair for 35s detection. To date only one transgenic variety has been approved for its production by the European Commission. In March 2010 '**Amflora'** (also known as **EH92-527-1**), a genetically modified potato cultivar developed by BASF Plant Science was approved for industrial applications in the European Union market. This GM potato was actually carrying NOS terminator (as most known potato GM clones), and not the 35s promoter in its construct (Holst-Jensen *et al.*, 2012); therefore, positive amplification only for NOS marker and not for 35s is logically expected for this potato transgene, and is exactly what I found in my results. Such results strongly suggest that transgenic potatoes are being sold in Spanish markets, but not declared. This may be surprising because the low acceptance of GM crops in Europe made BASF Plant Science decide to stop its

commercialization activities in Europe in January 2012. Moreover, in 2013, an EU court annulled the approval of BASF's Amflora, saying that the EU Commission broke rules when they approved the transgenic potato in 2010. Still, other potato modified varieties keep being developed and evaluated in the EU. Most of these studies are focused on pathogen resistance (Missiou *et al.*, 2004; Green *et al.*, 2012; Teagasc, 2012), since some of the potato diseases like potato "late blight" (*Phytphtora infestans*) or nematodes, keep difficult to control and are responsible for substantial losses for farmers.

Although this was a pilot study encompassing a few sample tested, it suggests that there is a need for more quality controls of this kind in the potato industry. Especially, when it refers to the Spanish potato industry, if we take into account that Spain is the largest GMOs grower of the EU (in 2012, over 120 thousand hectares of *Bt* maize were cultivated — 19.5 percent more than the previous year — representing 90 percent of GM crops in the EU, ISAAA Brief No. 44 - 2012). Only few GM crops have been approved by the European Commission, such as cotton, soybean and sugar beet varieties (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm) and it is a legal requirement that food products where the level of approved GMO exceeds 0.9% of unintentional adventitious presence are appropriately labeled (European Comission, 2003) in order that customers may make informed purchasing decisions. Notwithstanding it, illegal GMOs have been found for other crops and my results suggest that it may happen in potato too.

A reliable, easy-to-use, and effective tool for GMOs traceability seems to be not so easy to develop if we take into account the number of GMOs cultivated in field trials or for commercial production has constantly increased during the last two decades. So have also the number of species, the number of countries involved, the diversity of novel (added) genetic elements and the global trade. All of these factors contribute to the increasing complexity of detecting and correctly identifying GMO derived material (Holst-Jensen *et al.*, 2012). But, as said before, countries jurisdictions, consumers concerns and rights and public health oblige to make higher efforts in food and feed safety, even more in crops like potato, in which few practical progress have been made, to date.

7. CONCLUSIONS

7 CONCLUSIONS

1. The molecular markers evaluated for Marker Assisted Selection in the Potato Breeding Program of the company Appacale proved to be more efficient and cost effective than traditional methods used in Classical Breeding.
2. The molecular markers evaluated also proved to be useful for calculating the allele dosage of the parental in the Breeding Program.
3. SSRs markers proved to be a reliable, reproducible and easy-to-use method able to distinguish between all varieties in the company Germplasm Bank.
4. Presence of fraud and mislabeling in the Spanish potato market was detected using SSR markers.
5. Potential presence of non-declared transgenic potatoes has been detected using presence-absence tests for transgenes.

8. BIBLIOGRAPHY

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9. ANNEX:

PUBLISHED RESULTS

Food Control 26 (2012) 575–579

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Detection of mislabelling in the fresh potato retail market employing microsatellite markers

Claudia Lopez-Vizcón*, Felisa Ortega

Departamento de Biotecnología, APPACALE, S.A., Valle de Mena, 13, Burgos, Spain

ARTICLE INFO

Article history:
Received 12 May 2011
Received in revised form
14 February 2012
Accepted 15 February 2012

Keywords:
Mislabelling
Fraud
Potato
Microsatellites
Traceability

ABSTRACT

DNA analysis of fresh tubers collected in different years and locations in Spain under suspicion of fraud showed more than 50% of mislabelling. A second study with samples collected at random showed more than 20% of mislabelling. Mislabelling was detected both in samples with national and foreign origin, suggesting this problem could be not exclusive of the Spanish potato market. Two microsatellite markers (SSR) primer sets were used to compare the genotypes of the samples with the genotypes of stated controls. SSR showed to be a helpful, affordable and easy-to-use tool for food traceability.

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1. Introduction

Potato (*Solanum tuberosum*) is the fourth most important crop in the world and a staple part of many diets in several countries. In 2008, more than 329 million metric tones of potatoes were produced worldwide (FAOSTAT, 2011), more than 68 million metric tones only in the EU (FAOSTAT, 2011). In Spain, around 1.8 million metric tones of potatoes are consumed per year (MARM, 2010). The 65% of the potatoes consumed in Spain comes from foreign countries, mainly France (70% of the imports), U.K., The Netherlands, Germany and Morocco (MARM, 2010). Potatoes are consumed in Spain in very different ways: boiled, fried, baked, roasted and as the main ingredient of the Spanish omelette. Nowadays, there is a large number of potato varieties available in the market and most of them are classified depending on their taste, quality or end-use. Their prizes also depend on those characteristics.

Under current EU legislation there is a requirement for potatoes that are offered for wholesale or retailsale to be labelled with their variety name (Woolfe & Primrose, 2004). This is both for consumer protection and quality control. The general concern about healthy products and specially the competitiveness between producers have made essential the development of a reliable method of varietal identification (Woolfe & Primrose, 2004).

Traditionally, morphological traits such as leaf type, tuber shape or flower colour have been used to identify potatoes. However, these traits can be influence by many factors, like environmental ones, making this identification method lack in reliability and reproducibility (Rosa et al., 2010; Woolfe & Primrose, 2004). Moreover, once the potato is processed, varietal identification based on morphological traits will be extremely difficult, when not impossible. Other methods are based on isozymes and total protein extraction (Douches & Ludlam, 1991). Again, the results can be influenced by different factors, in this case, development stage and growth conditions of the plant. There are also molecular methods of identification. Random amplified length polymorphisms (AFLP) (Kim, Joung, Kim, & Lim, 1998) and short sequence repeat (SSR) or microsatellite markers (Ghislain et al., 2004; Mathias, Sagredo, & Kalazich, 2007; Mc Gregor, Greyling, & Warnich, 2000; Moisan-Thiery et al., 2005; Norero, Malleville, Huarte, & Feingold, 2002; Reid & Kerr, 2007) are the most common ones and have shown to be the most reproducible and reliable. The use of random amplification of polymorphic DNA (RAPD) markers is well suited to DNA fingerprinting (Dos Santos, Nienhuis, Skroch, Tivang, & Slocum, 1994; Thormann, Ferreira, Carmargo, Tivang, & Osborn, 1994), but difficulties in the reproducibility of the technique are a limiting factor for accurate analysis (Demeke, Sasikumar, Hucl, & Chibbar, 1997; Karp, Kresovich, Bhat, Ayada, & Hodgkin, 1997; Mc Gregor et al., 2000). Microsatellite markers are not only reproducible and

* Corresponding author. Tel.: +34 947298102; fax: +34 947298411.
E-mail addresses: info.tec@appacale.com, claudialopezvizcon@hotmail.com (C. Lopez-Vizcón).

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doi:10.1016/j.foodcont.2012.02.020

Table 1
Number of samples collected for each variety and years when they were sampled; A) samples collected under suspicion of mislabelling; B) samples collected at random.

Variety	Num. of samples	Years sampled
A)		
Monalisa	77	2003–2011
Caesar	26	2003–2008
Agata	21	2005–2008
Elodie	9	2005–2007
Nicola	3	2006
Agria	2	2008, 2010
Kennebec	2	2006
Liseta	2	2005
Lady Christl	1	2006
Blanka	1	2006
TOTAL	144	
B)		
Monalisa	8	2011
Agata	4	2011
Caesar	4	2011
Elodie	1	2011
Asterix	1	2011
TOTAL	18	

reliable, but very informative and simple to use, the method can be automated, and effective cost per genotype and analysis is lower than in other molecular marker technologies (Garcia et al., 2004; Moisan-Thiery et al., 2005). All these reasons justify DNA-based fingerprinting using SSRs as a reliable and efficient method for potato cultivar identification.

Despite EU and national legislations on food quality and control, some fraud and mislabelling problems have been detected in the Spanish food industry in the last years, specially in the fish market (Asensio, González, Pavón, García, & Martín, 2008; Garcia-Vazquez et al., 2011; Machado-Schiaffino, Martinez, & Garcia-Vazquez, 2008) however, the mislabelling levels for vegetables like potatoes is still quite unexplored. The aim of this study was to evaluate the mislabelling in the Spanish potato market using SSRs methodology for cultivar identification. Samples from different locations and supermarkets were analysed and the level of mislabelling was investigated.

2. Materials and methods

2.1. Plant material

A total of 144 samples consisting of a batch of 15–20 fresh tubers (3–5 kg) were collected from 2003 to 2011 by company clients in different locations and from different origins (supermarkets, distributors…). All the samples were under suspicion of fraud or mislabelling.

To evaluate the impact of this potential fraud in the Spanish market, other 18 samples consisting of a batch of 15–20 fresh tubers (3–5 kg) were collected in 2011 in seven of the top supermarket chains in Spain. The samples were collected at random in supermarkets from two different locations in northern Spain.

In all cases, 3 tubers were chosen at random for DNA analysis.

The number of samples of each potato variety sampled (Table 1. A and B) ranged between 1 (Lady Christl, Blanka and Asterix) and 77 (Monalisa) in the samples collected from 2003 to 2011, and between 1 (Axterix, Elodie) and 8 (Monalisa) in the samples collected at random in 2011.

42% of the samples collected between 2003 and 2011 had national origin, while a 28% had foreign origin, mainly France (90% of the foreign samples) (Fig. 1. a). Origin of the rest of the samples was unknown. Almost 28% of the samples collected at random in 2011 had a national origin. The rest were all from foreign origin (France) (Fig. 1. b).

2.2. DNA extraction

DNA was extracted from the skin of the tuber in the case of the collected samples according to Edwards, Johnstone, and Thompson (1991). DNA extraction from stated and control varieties was

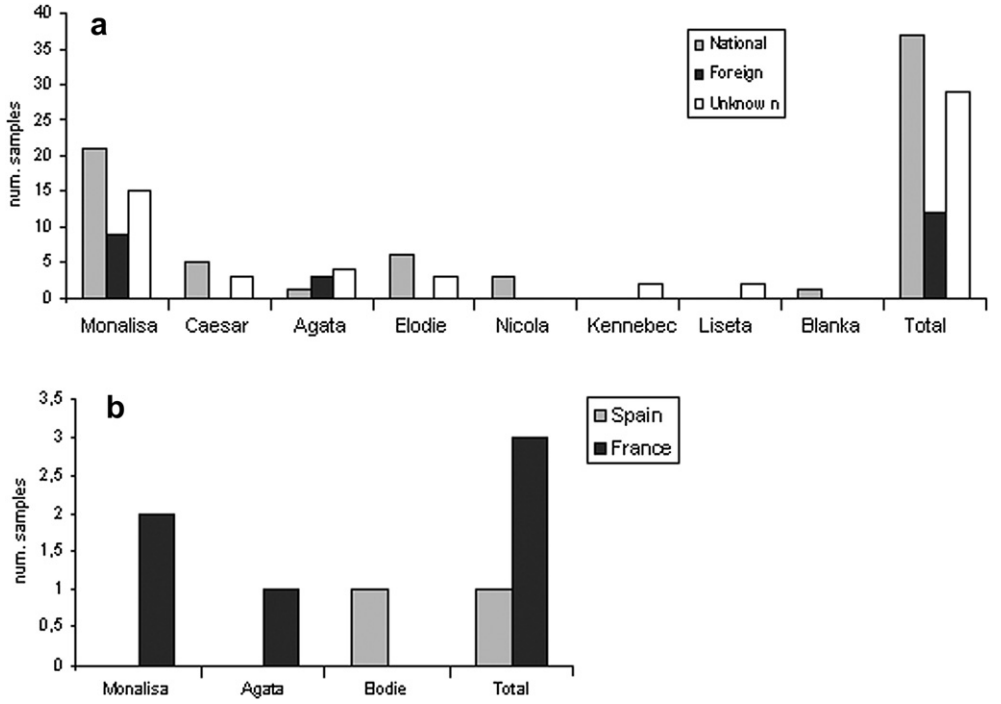

Fig. 1. Origin of the mislabelled samples for each variety and in total. A) Samples collected under suspicion of mislabelling (2003–2011). B) Samples collected at random (2011).

performed using tuber skin of leave tissue, according to the same protocol. 6 mg of fresh tissue were ground and collected in 400 μl of extraction buffer (0.2 M Tris (pH 7.5); 0.25 M NaCl; 0.025 M EDTA; 0.5% SDS). The tube was shaken in vortex and incubated at room temperature for 30 min. After centrifuging for 1 min at 13000 rpm, the supernatant was transferred to a new tube. 320 μl of isopropanol were added for precipitation and after centrifuging for 5 min at 13000 rpm and drying, the pellet was recovered in 400 μl of TE (10 mM Tris–HCl, pH 7.4; 1 mM EDTA, pH 8). All DNA extractions were repeated twice.

2.3. SSR methodology

SSRs were selected from previous studies that identified SSRs showing high levels of polymorphism in tetraploid potato (Milbourne et al., 1998; Provan, Powell, & Waugh, 1996). The two primer pairs used in this study are listed in Table 2.

PCR was carried out in a total reaction volume of 10 μl containing 10X PCR buffer [500 mM KCl, 100 mM Tris–HCl pH 8.3, 15 mM $Mg(OAc)_2$], 200 μM dNTPs, 0.5 μM each primer, 0.5 U *Taq* DNA polymerase (Bioron GmbH, Ludwigshafen, Germany) and 2 μl of DNA template DNA. All amplifications were carried out in an Applied Biosystems (Foster City, CA, USA) 9700 thermo cycler using the following parameters: 94 °C for 1 min; 94 °C for 30 s, [*Tm*] for 30 s, 72 °C for 1 min × 44 cycles; 72 °C for 15 min, (for *Tm* values refer to Table 2). PCR products were denatured by the addition of 10 μl stop solution (95% formamide) and heating to 94 °C for 5 min. Then 3 μl of each sample were loaded onto 6% polyacrilamide denaturing gels (8 M urea) buffered with 1X TBE and separated for 1–2 h at 90 W constant power using a DNA sequencing gel electrophoresis apparatus (BioRad, Hercules, CA, USA). Gels were stained with the method of Bassam, Caetano-Anollés, and Gresshoff (1991) modified as follows: gels were fixed for 45 min in 10% acetic acid; washed with distilled water for 5 min (2X); stained for 45 min in freshly prepared 0.1% $AgNO_3$, with 1.5 ml of formaldehyde; rinsed for 4–5 s with distilled water; developed for about 5 min in freshly prepared 3% Na_2CO_3 with 1.5 ml of formaldehyde added immediately prior to use; and fixed for 5 min in 10% acetic acid.

The genotype of each sample was compared with the genotype of stated and control varieties by amplifying them in the same PCR and then running them in the same gel. PCR was performed twice for each sample. The 3 tubers of each sample were analysed separately. Stated and control varieties were obtained from a Potato Genebank (NEIKER Potato Germplasm Bank, Spain) or from the correspondent Breeder (C. Meijer CV, HZPC Holland B.V.), which assures their authenticity.

3. Results

The two primer set employed in this work, allowed to distinguish between the eleven different varieties of potato analysed, establishing a unique pattern for each variety (Fig. 2).

Table 2
Primer information of microsatellites used for tetraploid potato variety identification.

Locus	Motif repeat		Primer sequence (5′–3′)	Tm (°C)
STM2020[a]	$(TAA)_6$	F	CCTTCCCCTTAAATACAATAACCC	60.2
		R	CATGGAGAAGTGAAAACGTCTG	59.8
STIIKA[b]	$(T)_{12}$ $(A)_9$ ATTCTTGTT	F	TTCGTTGCTTTACCTACTA	50
	$(TA)_2$ CA $(TA)_7$	R	CCCAAGATTACCACATTC	50

Primer/locis, sequence and repeated motif acquired from published literature.
[a] Milbourne et al. (1998).
[b] Provan et al. (1996).

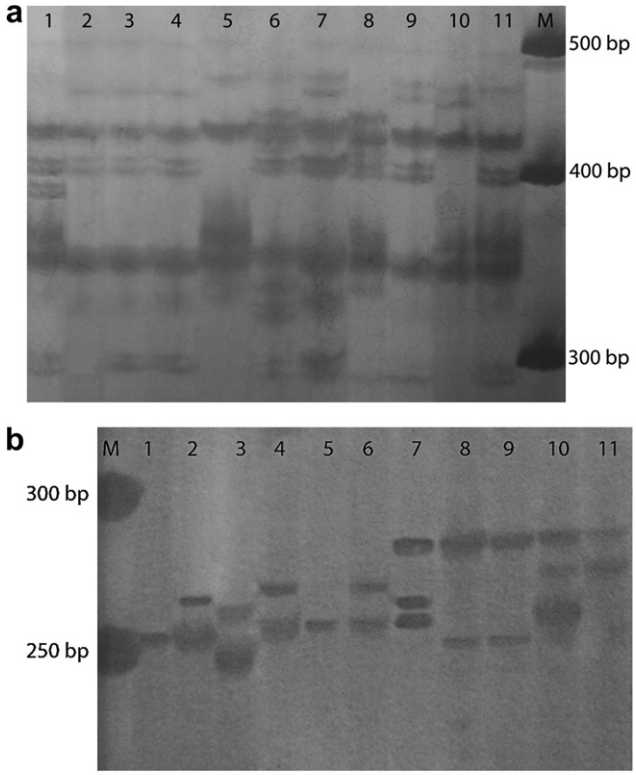

Fig. 2. SSR profiles of the *S. tuberosum* cultivar controls. a) for loci STIIKA (Provan et al. 1996) 1–11: 1, Lady Christl; 2, Kennebec; 3, Caesar; 4, Monalisa; 5, Elodie; 6, Asterix; 7, Agata; 8, Nicola; 9, Liseta; 10, Blanka; 11, Agria; M, Molecular weight ladder in base pairs (bp). b) for loci STM2020 (Milbourne et al. 1998) 1–11: 1, Caesar; 2, Elodie; 3, Asterix; 4, Agria; 5, Liseta; 6, Kennebec; 7, Lady Christl; 8, Monalisa; 9, Blanka; 10, Agata; 11, Nicola, M, Molecular Weight Ladder in base pairs (bp).

3.1. Samples collected under suspicion of fraud

High proportion of the total samples were amplified successfully (about 99%) with both pairs of primers and compared with the authenticated references. In the samples from 2003 to 2011 (under suspicion of fraud or mislabelling; Table 1. A.), 10 different varieties were analysed with different number of samples: from 1 (Lady Christl and Blanka) to 77 (Monalisa). Number of mislabelled samples ranged between 0 (Lady Christl and Agria) and 45 (Monalisa) (Fig. 3a). Total number of mislabelled samples was 78 out of 144 (54.2%). In the 98.4% of the cases, mislabelling was established for the 3 tubers analysed in each sample. In the remaining 1.6% (1 case) mislabelling was established for 2 of the 3 tubers analysed in the sample. A 6.2% of the mislabelled samples showed more than one genotype not corresponding with the labelled variety. 37 of the mislabelled samples (45.7%) were from national origin, while 13 (16.0%) had foreign origin (Fig. 1. a).

3.2. Samples collected at random

High proportion of the total samples were amplified successfully (about 99%) with both pairs of primers and compared with the authenticated references. In the samples collected at random in different supermarkets, in 2011 (Table 1. B.), 5 different varieties were analysed with different number of samples: from 1 (Elodie and Asterix) to 8 (Monalisa). Number of mislabelled samples ranged between 0 (Asterix and Caesar) and 2 (Monalisa) (Fig. 3. b). Total number of mislabelled samples was 4 out of 18 (22.22%). In the 75% of the cases of mislabelling, it was established for the 3

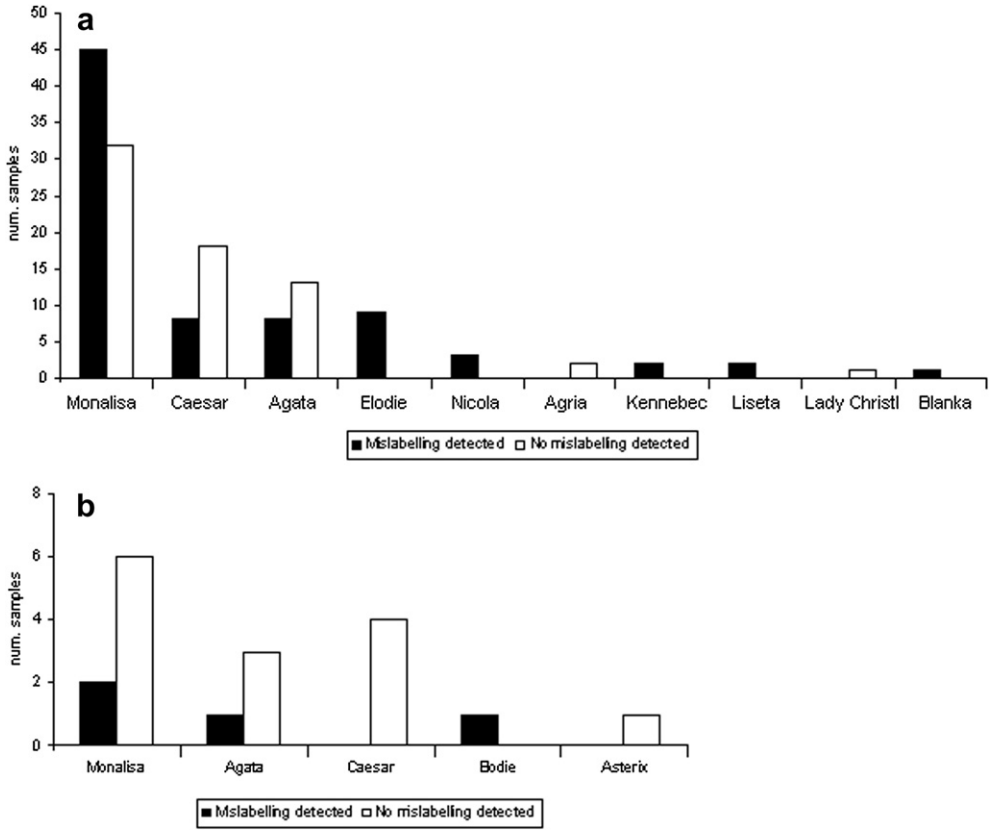

Fig. 3. Number of samples showing mislabelling for each variety. a) Samples collected under suspicion of mislabelling (2003–2011). b) Samples collected at random (2011).

tubers analysed in each sample. In the remaining 25% (1 case) mislabelling was established for 2 of the 3 tubers analysed in the sample. A 25% of the mislabelled samples showed more than one genotype not corresponding with the labelled variety. 1 of the mislabelled samples (25.0%) was from national origin, while 3 (75.0%) had foreign origin (France) (Fig. 1. b).

3.3. Comparison between samples collected under suspicion of fraud and samples collected at random

The differences found between the mislabelling in the samples that were under suspicion of fraud and the mislabelling in those that were sampled at random were highly significant (Yates $\chi^2 = 0.0$, p-value $= 1$), being higher the mislabelling detected in the samples collected under suspicion of fraud than in those collected at random.

4. Discussion

In this study a 22.2% of mislabelling was detected for the 18 samples collected at random, which differs (Yates $\chi^2 = 0.0$, p-value $= 1$) to the percentage detected on the under suspicion of fraud samples (54.2%). That indicates that mislabelling in the Spanish potato market does exist, although it is not as high as the first studies using under suspicion samples showed. The difference in the mislabelling percentage may be not only because the first samples were collected under suspicion of fraud and the second ones were collected at random. It could also be due to the origin of the samples. The samples under suspicion of fraud were collected in supermarkets but also from distributors or independent/small chains, while the samples collected at random were all from supermarkets. It is expected that the mislabelling from these different sources can be very different as well. The results also show that this mislabelling is recurrent in different years and not just a punctual problem. The mislabelling levels detected in the potato market are a bit lower than the levels found in other Spanish food products, like it is shown in a recent study about mislabelling in hake, where 38.9% of mislabelling is detected (Garcia-Vazquez et al., 2011); but still, the levels are significant. Moreover, the employment of a larger primer set could may be allow detecting higher levels of mislabelling. Although many studies describing traceability methodologies for the Spanish food industry have been developed (e.g. Fajardo et al., 2006; Fernández-Tajes, Freire, & Méndez, 2010; Rojas et al., 2010), not many scientific studies have been focused on the evaluation of real mislabelling and fraud levels in the Spanish market. These kinds of studies provide useful and necessary information to choose the best strategies to avoid and prevent mislabelling.

Under current EU legislation, there is a requirement for potatoes that are offered for wholesale or retailsale to be labelled with their variety name (EU Directive 2003/89/EC). Consumers have the right to know what they are buying and consuming. Most potato varieties are classified and prized depending on their characteristics and end-use. Consumers may purchase a particular variety of potatoes for any of the above reasons. Thus, this fraudulent substitution is unfair for the consumers, because they pay for a product that they are not buying in reality.

This study is focused exclusively on the Spanish market, but the origin of the samples that were analysed was not only national; in total, 66% of the samples came from foreign origins, mainly France (92% of the samples with foreign origin). Moreover, on average, a 45% of the mislabelled samples were from foreign origin. This

could suggest that the same problem could be occurring in other countries as well. In 2003, the Food Standards Agency of the United Kingdom ran a similar study (www.food.gov.uk/multimedia/pdfs/fsis4403.pdf) with revealing results. They found a high percentage (33%) of samples without appropriate labelling. However, this percentage was much lower (2%) when analysing samples coming only from big supermarkets, while it was found that small stores or chains, market stalls and wholesale markets were responsible for most of the mislabelled samples. This study also confirms that mislabelling in potato could be a problem not only in Spain but in other European countries. The study of the Food Standards Agency of the United Kingdom not only showed a high mislabelling percentage in the British potato market, they also found a problem with one of the most appreciated varieties in UK (King Edward), with a high percentage of mislabelling in the wholesale or market stalls. We believe that something similar could be occurring in Spain with variety Monalisa, one of the most appreciated and demanded varieties in Spain (MARM, 2010). Most of the samples collected under suspicion of fraud belonged to this variety, and a high percentage of mislabelling was found for this cultivar in the samples that were under suspicion of fraud and also in those collected at random.

It is important to point out that, by the time that the samples were collected at random (January–February), imported potatoes are stored potatoes coming mainly from France. Taking into account the results already obtained, it would be interesting to develop a similar study along May and June, when early potatoes start arriving from southern Spain, Morocco or Israel. That could let us evaluate whether or not does mislabelling exist in potatoes coming from these countries and if so, we could compare the results with the ones obtained in this work. It would also give us a wider perspective about mislabelling in the potato market not only in Spain or the EU, but in other important world potato producers.

This study was carried out using SSRs as an effective and reliable genetic tool for cultivar identification. Molecular markers have proved to be a helpful, affordable and easy-to-use tool for food traceability; this should encourage administrations to increase routine controls in order to avoid fraud and ensure consumers rights. From our results and from other studies in different products and markets, it is evident that more efforts should be done in traceability and food control in Spain and in the rest of the EU, and genetic tools give the possibility to enforce all these actions.

Acknowledgements

We want to thank Dr. J.L. Horreo for his help, advice and text revision.

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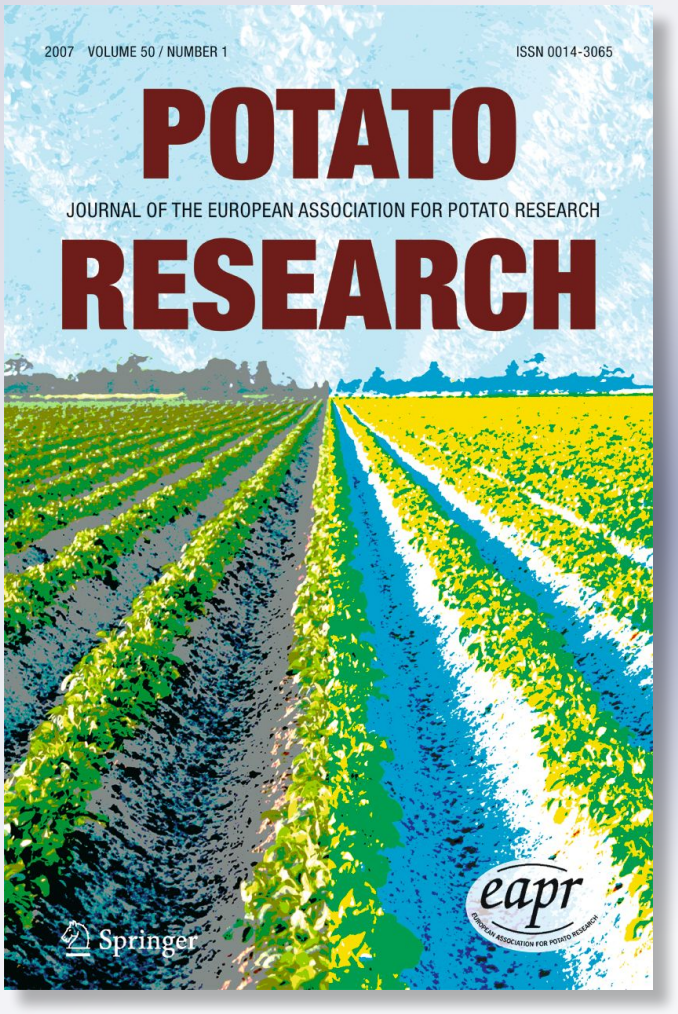
Application of Molecular Marker-Assisted Selection (MAS) for Disease Resistance in a Practical Potato Breeding Programme

Felisa Ortega & Claudia Lopez-Vizcon

Potato Research
Journal of the European Association for Potato Research

ISSN 0014-3065

Potato Res.
DOI 10.1007/s11540-011-9202-5



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Potato Research
DOI 10.1007/s11540-011-9202-5

Application of Molecular Marker-Assisted Selection (MAS) for Disease Resistance in a Practical Potato Breeding Programme

Felisa Ortega · Claudia Lopez-Vizcon

Received: 25 March 2011 / Accepted: 2 December 2011
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Abstract The company Appacale started applying molecular markers in 1998 with the implementation of RYSC3 marker of resistance to Potato virus Y (PVY). Since then, five more molecular markers have been implemented, and now it is possible to select for PVY, *Globodera rostochiensis* and *Globodera pallida* resistance with this technology. The markers used have allowed the selection of breeding clones with resistance to one or more pathogens that are currently part of the breeding programme, new cultivars or even being used as resistant parents. The results obtained are presented, as well as the advantages and setbacks found in applying Molecular-Assisted Selection from the point of view of a practical breeding programme developed in a small company.

Keywords Breeding · *G. pallida* · Gro1-4 · *G. rostochiensis* · HC · Molecular markers · PVY · RYSC3 · SPUD1636 · STM0003 · TG689

Introduction

Despite the large number of new potato (*Solanum tuberosum* L.) cultivars currently available, there is still a need for new ones. The potato industry in the European Union is trying to increase potato handling in an economically and environmentally sustainable way. New cultivars must give economic benefits through more yield of saleable product at less cost of production, with reduced disease and pest attacks, and tolerance to environmental stresses. Developing a new variety can take up to 12 years from crossings to release, so improved breeding strategies are needed.

Molecular markers offer a striking promise for plant breeding. The opportunity to select desirable lines based on genotype rather than phenotype, analysing plants at the seedling stage, screening multiple characters, minimizing linkage drag and rapidly

F. Ortega (✉) · C. Lopez-Vizcon
Appacale SA, Valle de Mena, 13, 09001 Burgos, Spain
e-mail: fortega@appacale.com
URL: http://www.appacale.com/index.htm

Published online: 04 January 2012

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recovering a recurrent parent's genotype are just some of the attractions of Marker-Assisted Selection (MAS; Collard and Mackill 2008). However, it soon became apparent that applying knowledge gained through molecular mapping in real world breeding might not be entirely straightforward. The reality is that MAS has had only a limited impact on plant breeding so far (Young 1999; Collard and Mackill 2008).

Pests and diseases are the major threat to potato cultivation worldwide. Thus, durable disease and pest resistance is a primary goal of most potato breeding schemes. Classical breeding for resistance involves the identification of resistance sources, which are often found in wild and unadapted germplasm. Since 1990, many of these resistance factors have been located on the potato molecular linkage map using DNA-based markers. They have been mapped either as major genes (*R* genes) or as quantitative trait loci (QTL; review in Gebhardt and Valkonen 2001; Simko et al. 2007). The dominant gene for extreme resistance to Potato virus Y (PVY), Ry_{adg}, was identified in *S. tuberosum* ssp. *andigena* (Muñoz et al. 1975) and mapped to chromosome XI (Hämälainen et al. 1997). Kasai et al. (2000) developed RYSC3 polymerase chain reaction (PCR)-based marker that simultaneously functions for Ry_{adg} and the gene Na_{adg} of hypersensitive resistance to Potato virus A (PVA). Some years later, Song et al. (2005) mapped the Ry_{sto} gene to chromosome XII co-segregating with the molecular marker STM0003. Regarding potato cyst nematodes (PCN), the monogenic dominant *Gro1* gene from *Solanum spegazzinii* for resistance to all pathotypes of *Globodera rostochiensis* was mapped to the potato molecular map on chromosome VII (Barone et al. 1990). *Gro1-4*, a member of *Gro1* locus, showed to confer resistance to *G. rostochiensis* pathotype Ro1 (Paal et al. 2004). Besides, the most widely used dominant gene of resistance to *G. rostochiensis* Ro1 is gene *H1* from *S. tuberosum* spp. *andigena*, mapped to chromosome V (Gebhardt et al. 1993; Pineda et al. 1993). On the other hand, the most prominent and reproducible QTL for *G. pallida* resistance was mapped to chromosome V, and the diagnostic value for the linked DNA marker SPUD1636 was demonstrated by Bryan et al. (2002) in some accessions with *Solanum vernei* as source of resistance. Later on, Sattarzadeh et al. (2006) reported a PCR assay "HC" with higher diagnostic value for this QTL.

Disease resistant genotypes are often simple and oligogenic in nature, but the difficulties in establishing reliable inoculation methods and scoring can be a discouraging handicap. MAS can be extremely powerful in this field. But even so, published examples of the use of molecular markers for MAS of disease/pest resistance are mainly limited to diploid material and a small number of genes (Barone 2004; Ottoman et al. 2009).

Appacale is a public company located in Burgos (Spain) founded to support Spanish seed potato producers by the Government of Castilla-Leon and the main seed potato producer cooperatives of the autonomous region, and thus of Spain, as Castilla-Leon produces 72% of Spanish seed potato. The company performs breeding for all market segments present in Spain (fresh market and processing). The breeding programme is carried out as a classical one based on phenotypic selection, but since 1999, investments in applying new technologies, as molecular markers, have been made in order to improve these technologies, making the selection easier. The main priorities in the company breeding programme regarding disease resistance are

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viruses and PCN. These pathogens can cause great economic losses either directly as yield decrease or indirectly taking into account the costs of preventive or control treatments, quarantine laws for PCN or loss of economic value of seed potatoes due to PVY infection. The use of resistant cultivars is the most efficient protection against them. In this article, the work done by the company to select breeding clones resistant to these diseases applying molecular markers is presented. The advantages and setbacks found in applying MAS from the perspective of a practical breeding programme developed in a Small and Medium Enterprise (SME) are also pointed out.

Materials and Methods

Plant Material

Breeding clones evaluated for the presence of molecular markers were those of the breeding programme developed at the company which had as parent one or more resistant cultivars known to amplify any of the specific markers described later or a first backcross of these parents. Therefore Ry_{adg} genotypes used were: varieties 7XY-1, LT-8, LT-9, P-6, Tacna and V-2 and breeding clones 95APP-4 (Spunta × V-2), 95APP-5 (Atlantic × V-2), 95P17-3 and 95P17-7 (Iroise × V-2), 95P63-11 (Spunta × V-2), 95P87-4 (Frisia × V-2), 96YS51-1 (Atlantic × BR63-66), 96YS21-13 (LT-8 × Tobique), 97YS211-3 (Hertha × V-2), 97YS215-3 (LT-9 × Vanesa), 99YS181-10 (Bartina × Bulk), 99YS185-1 (Cinja × Bulk), 99YS188-3 (Hertha × Bulk), 99YS191-1 and 99YS191-8 (LT-8 × Hermes), and 99YS192-5, 99YS192-9 and 99YS192-12 (Maris Piper × Bulk); Ry_{sto} genotypes: Bzura, Forelle, Pirola, White Lady and breeding clones 94APP-2 (Forelle × Sandra) and 94APP-4 (Pirola × Leila); varieties resistant to *G. rostochiensis* with the *Gro1-4* gene Optima, Valetta and 94APP-3 (Optima × Valetta), and with *H1* Atlantic, Cara, Lady Claire, Santé and Saturna; and finally, varieties with resistance to *G. pallida* Pa2,3 Santé and Innovator. These varieties had been obtained in previous years from different breeding institutions or genebanks and maintained in the progenitor collection of Appacale.

Molecular Markers and Cost Estimation

Total DNA was extracted from fresh leaves according to Edwards et al. (1991). Six milligrams of fresh tissue was ground and collected in 400 μl of extraction buffer [0.2 M Tris (pH 7.5), 0.25 M NaCl, 0.025 M EDTA, 0.05% SDS]. The tube was shaken in vortex and incubated at room temperature for 30 min. After centrifuging for 1 min at 13,000 rpm, the supernatant was transferred to a new tube. Isopropanol, 320 μl, was added for precipitation, and after centrifuging for 5 min at 13,000 rpm and drying, the pellet was recovered in 400 μl of TE (10 mM Tris–HCl, pH 7.4; 1 mM EDTA, pH 8).

The Ry_{adg} and Ry_{sto} genes for resistance to PVY were identified using the SCAR marker RYSC3 described by Kasai et al. (2000) and marker STM0003 described by Song et al. (2005), respectively (Table 1). The *Gro1-4* locus of resistance to *G. rostochiensis* was selected by using specific primers for the *Gro1-4* resistance gene

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Table 1 Molecular markers applied in the company and total number of molecular marker analyses performed in the breeding clones of the company's programme since 2000 to select disease resistance

Pathogen	Gene	Chr	Marker	Reference	Years	Total analysed	Positive
PVY, PVA	Ry_{adg}, N_{adg}	XI	RYSC3	Kasai et al. (2000)	2000–2010	1,197	451
PVY	*Rysto*	XII	STM0003	Song et al. (2005)	2006–2010	536	233
G. rostochiensis	*Gro1-4*	VII	Gro1-4	Gebhardt et al. (2006)	2008–2010	43	15
G. rostochiensis	*H1*	V	TG689	W.S. De Jong (personal communication)	2010	6	2
G. pallida	*RGpa5*	V	SPUD1636	Bryan et al. (2002)	2010	6	1
G. pallida	*RGpa5-vrnHC*	V	HC	Sattarzadeh et al. (2006)	2007–2010	152	76
Total						1,940	778

adapted from Gebhardt et al. (2006). The TG689 protocol to select the *H1* gene of resistance to *G. rostochiensis* was kindly provided by W.S. De Jong. Thus, PCR was performed in a total volume of 20 μl containing 0.4 μl 10 μM of TG689 allele specific and TG689 indel12 primers, 0.2 μl of primers DCH-F2 and 10 μM DCH-R2, 2 μl 10× PCR buffer, 1.6 μl 2.5 mM dNTPs, 2 μl DNA and 0.2 μl 5U/μl DFS-Taq DNA polymerase (Bioron GmbH). PCR conditions were: initial denaturation for 2 min at 94 °C, 35 cycles of 20 s at 94 °C, 30 s at 55 °C and 3 min at 72 °C, and one cycle of final extension for 5 min at 72 °C. PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. SPUD1636 for *G. pallida* resistance was implemented from Bryan et al. (2002) and the HC marker assay is described by Sattarzadeh et al. (2006). Markers were applied on the second year of field trials of the breeding clones.

The cost of applying any of the mentioned markers to one genotype was estimated considering that about 30 assays are usually performed at the same time. Thus, total labour time for DNA extraction, PCR and electrophoresis was estimated and then divided by 30 to know how many minutes corresponded to each genotype, and then translated into euros according to the cost of 1 h of work of the personnel involved. Costs of primers, Taq polymerase and agarose or acrylamide were specifically calculated for each primer pair. A common amount of 0.72 € for fungible material as tubes, tips, buffers, etc. was added to all of them. Indirect costs were not considered.

Validation of STM0003 Marker

To test if the marker STM0003 identified the PVY resistance, all the breeding clones that were positive for the STM0003 marker and were selected in their second year of field trials because of their good agronomic performance (the same year that molecular markers are applied, see above) were tested the following year (third year of field trials)

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on two different dates for PVY infection with ELISA-DAS, from leaves collected in the field trials.

Cost Estimation of PVY Artificial Inoculation

Costs of artificial PVY inoculations were estimated for the standard procedure that is usually used at the company (inoculation tests were not performed). According to this protocol, three virus-free tubers per genotype are grown in 10-cm pots in a greenhouse at 15 °C (range, 15–30 °C) with natural long-day summer light (April–September). Once grown (about 6 weeks later), plants are infected with sap from tobacco fresh leaves checked for infection with PVY^{o} or PVY^{NTN}. Symptoms are recorded and all plants tested at least once by enzyme-linked immunosorbent assay (ELISA) about 4 weeks after inoculation. Plants that exhibit visual symptoms or/and give a positive ELISA result would be considered as susceptible and symptomless response and negative ELISA results as resistant. Inoculations are repeated the second year in all genotypes considered as resistant to confirm their resistance.

Labour costs of planting, watering, inoculation, ELISA tests, etc. per genotype were calculated, plus material costs like pot soil and ELISA antiserums. Indirect costs were not included.

Cyst Nematode Artificial Inoculation and Cost Estimation

Artificial inoculations to test nematode resistance were developed following the procedure described by the Council Directive 2007/33/EC. Thus, the tests were performed in climate chambers under controlled conditions of light and humidity. Variety Désirée was used as susceptible standard and *G. rostochiensis* population Ecosse as inoculum to test Ro1 resistance. The inoculum consisted of a total of five infective eggs per millilitre of soil. We used six replicates per variety. Plants were cultivated during 3 months. Cysts from the six replicates were extracted with a Fenwick can and counted separately for each pot. The relative susceptibility of the tested varieties or breeding clones was expressed as a percentage related to the final population of the susceptible control and scored from 9 (resistant) to 1 (susceptible).

Labour costs of preparing the inoculums, planting, watering, washing sand, counting cysts, etc. per genotype were calculated. Indirect costs were not included.

Segregation Ratios of the Progenies

The total analyses performed in the progenies descending from the same parent (variety or breeding clone) used as resistance donor were pooled, and the segregation ratios of the markers in those progenies compared with the expected ratios of chromosome (simplex, 1:1 resistant/susceptible; duplex, 5:1; triplex, quadruplex 1:0) and chromatid segregation (simplex, 0.86:1 resistant/susceptible; duplex, 3.67:1; triplex, 27:1; quadruplex, 1:0) with a chi-square goodness-of-fit test with the Yates correction for continuity, according to Zar (1974). Only progenies with more than 100 tests per parent were included in the analysis.

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Results

Since 2000, 1,940 tests were performed on the progenies that could amplify markers RYSC3, STM0003, Gro1-4, TG689, SPUD1636 and HC based on their pedigree; 778 (40.1%) of them amplified any of the markers and were regarded as resistant (Table 1). Some of them are still maintained as advanced breeding material or are actually being tested in the official registration assays to be released as new varieties. Among them, 1,197 genotypes were tested with RYSC3 marker, 451 (37.7%) of them being positive for the marker and 140 (11.7%) selected in their second year of field trials for further selection (Table 2). Seven of these clones are still maintained at the breeding programme as breeding material. Besides, since 2006, 536 assays have been

Table 2 Molecular marker analysis performed to test the presence of marker RYSC3 for PVY resistance since 2000, number and percentage of resistant clones selected in the second year of field trials and still at the breeding programme (early clones, 3rd- to 4th-year clones; medium, 5th- to 6th-year clones and advanced ones, 7th-year or more or registered varieties)

Parent	Total analysed	Positives (resistant)	Selected (2nd year)		Currently at the breeding programme		
			N	%	Early	Medium	Advanced/registered
7XY-1	3	1	0	0.0	0	0	0
LT-8	85	38	12	31.6	0	0	0
LT-9	90	36	9	25.0	0	0	0
P-6	39	7	2	28.6	0	0	0
TACNA	37	15	3	20.0	0	0	0
V-2	136	78	33	42.3	0	0	0
95APP-4	17	7	1	14.3	0	0	0
95APP-5	152	57	18	31.6	1	0	0
95P17-3	63	24	7	29.2	0	0	0
95P17-7	30	12	3	25.0	0	0	0
95P63-11	117	31	8	25.8	1	0	0
95P87-4	114	46	12	26.1	1	0	0
96YS51-1	42	11	2	18.2	1	0	0
96YS21-13	6	1	0	0.0	0	0	0
97YS211-3	26	11	2	18.2	0	0	0
97YS215-3	22	9	5	55.6	0	1	0
99YS181-10	11	2	2	100	0	0	0
99YS185-1	15	5	2	40.0	0	0	0
99YS188-3	8	2	1	50.0	0	0	0
99YS191-1	26	3	1	33.3	1	0	0
99YS191-8	30	20	5	25.0	0	0	0
99YS192-5	33	14	1	7.1	0	0	0
99YS192-9	26	14	7	50.0	0	0	0
99YS192-12	69	7	4	57.1	0	0	1
Total	1,197	451	140	31.0	5	1	1

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performed with STM0003 marker to select PVY resistance conferred by Ry_{sto} gene, 233 (43.5%) of them being positive and thus regarded as resistant (Table 3). Fifty nine of these clones (11.0%) were selected because of their good agronomic performance, and seven of them are still in the breeding programme. However, adding up the results of 2006 to 2010, we found that 5 of these 59 breeding clones that amplified STM0003 were positive for PVY infection the year after the marker test was performed.

The presence of *Gro1-4* locus of *G. rostochiensis* Ro1 resistance was evaluated in 43 breeding clones, 15 of them being positive (34.9%; Table 4). Seven of them (16.3%) were selected because of their good agronomic performance, and two of them are advanced material. On the other hand, varieties that could have *H1* gene and six breeding clones were checked for the presence of the RFLP marker TG689 in 2010, with all the varieties and two of the breeding clones being positive (33.3%; Table 1). Also, 16 breeding clones analysed with markers Gro1-4 and TG689 were inoculated with *G. rostochiensis* Ro1 cysts (Table 5). All the resistant clones amplified markers Gro1-4 or TG689 according to their pedigree, and susceptible ones did not.

Finally, 158 genotypes were tested with markers SPUD1636 or HC for *G. pallida* Pa2,3 resistance. Seventy seven of them had the QTL of resistance (48.7%; Table 1).

Regarding the economical costs of MAS, the costs of phenotyping with artificial inoculations for PVY and cyst nematodes were compared with the costs of applying each marker (Table 6). Molecular markers were less expensive if the resistance could be selected with one or two diagnostic markers.

In addition, the segregation ratios of the markers in those progenies with more than 100 genotypes analysed per parent were compared with the expected ratios of chromosome and chromatid segregation. Results are shown in Table 7. In four of the six parents analysed (66.7%), there were no significant differences between the ratios observed and expected for the simplex dosage, either with chromosome or chromatid segregation, but in two cases, the progenies did not fit any expected ratio.

Table 3 Molecular marker analysis performed to test the presence of STM003 marker for PVY resistance, number and percentage of resistant clones selected in the second year of field trials and still at the breeding programme (early clones, 3rd- to 4th-year clones; medium, 5th- to 6th-year clones and advanced ones, 7th-year or more or registered varieties)

Parent	Total analysed	Positives (resistant)	Selected (2nd year)		Currently at the breeding programme		
			N	%	Early	Medium	Advanced/registered
BZURA	28	13	3	23.1	0	0	0
FORELLE	31	11	3	27.3	1	0	0
PIROLA	78	26	0	0.0	0	0	0
WHITE LADY	352	163	48	29.5	2	4	0
94APP-2	42	17	4	23.5	0	0	0
94APP-4	5	3	1	33.3	0	0	0
Total	536	233	59	31.2	3	4	0

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Table 4 Molecular marker analysis performed to test the presence of Gro1-4 marker of *G. rostochiensis* resistance, number and percentage of resistant clones selected in the second year of field trials and still at the breeding programme (early clones, 3rd- to 4th-year clones; medium, 5th- to 6th-year clones and advanced ones, 7th-year or more or registered varieties)

Parent	Total analysed	Positives (resistant)	Selected (2nd year)		Currently at the breeding programme		
			N	%	Early	Medium	Advanced/registered
OPTIMA	2	1	0	0.0	0	0	0
VALETTA	2	0	0	0.0	0	0	0
94APP-3	39	14	7	50.0	1	0	2
Total	43	15	7	46.6	1	0	2

Discussion

Information about the validation and practical application of the discovery and mapping of qualitative and quantitative genes conferring resistance to important diseases and pests affecting potato in the form of MAS in practical breeding programmes has not been very common so far. RYSC3 SCAR marker was the first applied and validated in a private company as reported by Ruiz de Arcaute et al. (2002) in Appacale's Breeding Programme; afterwards, no more phenotyping and

Table 5 Results of the molecular marker analysis and inoculation tests performed for *G. rostochiensis* resistance in some breeding clones and varieties

Breeding clone	Female parent	Male parent	Marker[a]	Level of resistance
2000P4-6	94APP-3	Caesar	Gro1-4+	R
2000P21-2	Caesar	Valetta	Gro1-4-	S
2000YT132-13	Szignal	Valetta	Gro1-4+	R
2001P24-11	94APP-3	Caesar	Gro1-4+	R
2002P7-1	94APP-3	CA(7-10)	Gro1-4-	S
2004P3-52	94APP-3	Caesar	Gro1-4+	R
2004P3-10	94APP-3	Caesar	Gro1-4+	R
2004Q3-3	94APP-3	Caesar	Gro1-4+	R
2005Q49-3	94APP-3	Caesar	Gro1-4+	R
2006P30-9	94APP-3	Caesar	Gro1-4+	R
2000Q84-7	Helena	Atlantic	TG689+	R
2001Q29-10	94APP-4	Atlantic	TG689-	S
2001Q29-4	94APP-4	Atlantic	TG689+	R
2002P67-6	94APP-2	Atlantic	TG689-	S
2004P13-13	Lady Claire	Caesar	TG689-	S
2004P13-6	Lady Claire	Caesar	TG689-	S

R resistant, *S* susceptible in the inoculation tests

[a] Gro1-4+ and TG689+ means positive for the marker

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Table 6 Costs of applying each of the markers implemented in the company, compared with the costs of artificial inoculations of each pathogen

Pathogen	Marker		Artificial inoculation
PVY	RYSC3	2.83 €	6–12 €[a]
	STM0003	2.88 €	
G. rostochiensis	Gro1-4	2.84 €	38.6–77.2 €[a]
	TG689	3.10 €	
G. pallida	HC	2.84 €	
	SPUD	3.10 €	

[a] The lower amount corresponds to 1 year of inoculation (susceptible genotypes). The higher amount corresponds to a second year of inoculation to confirm if the putative resistant genotypes are really resistant

validation work have been done. Also, recently, Ottoman et al. (2009) have reported the validation and implementation of this marker in the US Pacific Northwest Potato Breeding Programme. PVY resistance from the wild species *Solanum stoloniferum* (Ry_{sto}) has been selected with STM0003 marker (Song et al. 2005). We found that the 8.3% of the selected clones regarded as resistant (because they amplified the marker), were infected with PVY, according to ELISA test. This result may be due to errors in ELISA or PCR assays or to recombination. In any case, the marker was able to identify effectively almost 92% of the resistance, which we consider justifying its use as a tool to select the PVY resistance in the breeding programme. Regarding PCN resistance, according to the artificial inoculations performed, Gro1-4 and TG689 were suitable for the breeding programme since all the clones tested phenotypically showed levels of resistance according to their genotypes, but although no recombination event was observed, the number of genotypes tested was too small to reliably assess the frequency of false positives due to recombination. Gebhardt et al. (2006) also found a total correspondence among Gro1-4 amplifying genotypes and their phenotypes, supporting the suitability of this marker as a diagnostic tool.

Table 7 Analysis of the segregation ratios of the markers in some of the progenies, compared with the expected ratios of chromosome and chromatid segregation by a χ^2 test

Parent	Gene	No. of tests	Inferred dosage	χ^2	
				Chromosome segregation	Chromatid segregation
V-2	Ry_{adg}	136	Simplex	2.6	6.7**
95APP-5	Ry_{adg}	152	–	9.0	4.7*
95P63-11	Ry_{adg}	117	–	24.9**	18.4**
95P87-4	Ry_{adg}	114	Simplex	3.8	1.6
WHITE LADY	Ry_{sto}	352	Simplex	1.78	0.0
INNOVATOR	*Gpa5*	152	Simplex	0.01	0.8

– the observed segregation ratio matches none of the possible allele dosages

*p value <0.05; **p value <0.01

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On the other hand, the diagnostic value for DNA markers SPUD1636 and HC linked to *G. pallida* resistance was demonstrated by Bryan et al. (2002) and Sattarzadeh et al. (2006), respectively. Both markers have been used for *G. pallida* selection. However, no phenotyping work has been performed to validate this marker, as inoculation tests for *G. pallida* are often ambiguous and also because, in this case, the markers are diagnostic for a QTL, not a major gene. The authors trusted the information provided by Bryan et al. (2002) and Sattarzadeh et al. (2006). The deployment of effective resistance against *G. pallida* is difficult because of its genetic complexity. In this situation, the availability of two markers that identify a great amount of the genetic resistance is highly valuable for the breeding programme, even if no complete resistance is achieved.

Besides, an additional benefit of molecular markers is the possibility to know the allele dosage of the resistance gene of each parent based on the segregation ratios of the markers in the progenies. All the parents analysed have the gene of resistance in simplex, but some of them could have had it in duplex or even triplex. This is very interesting because it is possible to construct superior parents with multiple copies and thus obtain full-sib resistance progeny. Currently, the PCR assays used are not dosage sensitive, so we depend on progeny testing to determine the number of resistant alleles present in a particular parent. But even so, progeny testing is simpler, quicker and cheaper with molecular markers than with artificial inoculations. Recently, a specific study to know the allele dosage of some of the parents used in the company as donors of resistance to *G. rostochiensis* linked to Gro1-4 marker has been performed, identifying one parent with the gene in duplex dosage (López-Vizcón and Ortega 2011). In the other cases evaluated, the progenies did not fit any expected ratio. This could be due to errors in the marker assays or more probably in the crossings performed to obtain the breeding clones. For example, in the case of the breeding clone 95APP-5, when it was used as female, chi-square values fit a simplex dosage ($\chi 2_{\text{chromosome}}=3.11$, $\chi 2_{\text{chromatid}}=1.72$, $N=63$), but when it was used as pollinator, the dosage is not clear ($\chi 2_{\text{chromosome}}=5.44^{*}$, $\chi 2_{\text{chromatid}}=3.04$, $N=89$). This could indicate undesired pollinations or errors in the classification of berries in some of the crossings performed to obtained seeds with 95APP-5 as pollinator.

The breeding programme carried out in Appacale follows the traditional scheme of crossings, seedlings, first year of field trials in a one-tuber plot per genotype at seed site, second year of field trials in a seven-tuber plot at seed site, etc. In this system, there is a huge selection pressure in the first years of clonal selection in the field based only on general appearance and agronomic performance, giving a reduction of about 85–90% of the starting material. PVY and *Globodera* spp. markers are applied in the second year of field trials, after the first year sieve, because the breeding process is a permanent counterbalance between costs and benefits; applying the markers in the first year, when most of the material will be discarded, would be too expensive and not efficient at all. Moreover, breeding clones analysed with molecular markers are selected based on their phenotype plus their genotype. That means that being positive for the marker is considered as an important plus, but not all of the positive ones are selected and not all the negative ones are discarded. All the evaluated genotypes have to accomplish a minimum of agronomic performance to pass to the following year of selection. This is an important point because there are some varieties that produce progenies that can be selected with molecular markers but that produce very "bad"

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descendants. This is an important limit for MAS, as will be discussed later. MAS in the seedling generation is too costly due to the large number of DNA extractions and PCR assays required. For example, in 2010, 26,184 seeds were sown at the seedlings step, and 14,833 clones were planted in the first year of field trials; 9,021 and 8,118 of them, respectively, could have amplified any of the markers. Assuming that about 90% of the seeds were able to germinate and there were losses due to sprouting problems or others in the field, about 7,300 or 8,000 analyses should have been done, respectively. On the contrary, 225 marker analyses were performed in the second year breeding clones. Translated into money, multiplying each analysis per a mean amount of 3 € (see Table 6), the costs vary from 21,900 € at the seedlings step or 24,000 € in the first year of field trials to 675 € in the second year of field trials. Thus, although it has been traditionally said that molecular markers are too expensive to be systematically used, if they are applied in the suitable step of a breeding programme (that should be decided by each company or institution), it is obvious that molecular markers allow an early selection of resistant clones in an affordable way. Also, comparing the costs of molecular markers and artificial inoculations, markers provide an earlier, quicker, cheaper and more reliable system of screening for the resistances. This is true only for published markers; developing new ones is not affordable nowadays for a small breeding company like Appacale.

Added to other well-known benefits of MAS, another advantage of special interest for breeding is the possibility to pyramidize genes in one genotype as demonstrated by Gebhardt et al. (2006). At this moment, the company has four breeding clones with more than one resistance that are being used as parents. Our progress is not as speedy as reported by Gebhardt et al. (2006) firstly because, until 2006, only RYSC3 marker could be applied at the company and, secondly, because at least 1 year of field selection is performed before applying the marker. Thus, the minimum time needed until a genotype is used as parent is 4 years (crossings, seedling, 2 years of field trials). An additional problem for practical breeding is that not all the genotypes are able to flower, so they have no use as parents even if they have multiple resistances. In other cases, the resistance is linked to male sterility, as in the case of Ry_{sto} resistance (Ross, 1986), and they can only be used as female parents, limiting their use.

It has been shown that the benefits and usefulness of MAS to select interesting resistances are many. Despite this, MAS has been so far of limited relevance in commercial potato breeding programmes due to different handicaps. Probably, the most important of them is that markers can only be applied to certain genetic background, and therefore, there are still large amounts of resistance that cannot be selected with this procedure. For instance, nowadays in Appacale, there are 32 highly PVY-resistant parents in the progenitor collection, according to our own information and/or the European Cultivated Potato Database (www.europotato.org), but only six (19%) and nine (28%) of them have the Ry_{adg} or Ry_{sto} genes, so their progenies could be selected with RYSC3 or STM0003 markers, respectively. The same holds for *G. rostochiensis*: there are 50 parents resistant to pathotype Ro1-4 in the company's genebank, but only 11 of them amplify Gro1-4 (22%) and 5 TG689 (10%). In the case of *G. pallida*, the sources of resistance are really scarce; there are only four resistant varieties, and three of them could be selected with SPUD1636 or HC markers. Additionally, some of the varieties that can be selected with markers produce progenies that could be resistant but have very low agronomic value. This is because,

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by reason of feasibility, linkage analysis of quantitative and qualitative agronomic traits in potato using DNA-based markers is mostly being performed in experimental, diploid or interspecific mapping populations, not well adapted and with fewer general agronomic qualities than advanced breeding clones or cultivars. This is shown by the Ry_{adg} selection rates in Table 2. In this table, it can be seen that the level of selection of clones obtained from Ry_{adg} varieties is near or slightly superior to the mean value of the general breeding programme for that year of field trials, which varies from 17% to 25% of the clones (data not shown). However, none of these breeding clones "has gone further" than the third year of field trials; they have not been successful at all. Ry_{adg} varieties 7XY-1, LT-8, LT-9, P-6 or V-2 were bred by the International Potato Centre (Lima, Peru), and they are not well adapted to European long-day conditions, so they usually produce progenies with dormancy problems, among others, that make them not suitable for European conditions. This tendency is reduced to some extent when the first backcrosses of these varieties are used as resistance donors (99YS191-1, 99YS191-12, etc.), but even so, their success is low. In the case of Ry_{sto}, it is not possible to compare among varieties because there are few data out from the Hungarian variety White Lady; however, the progenies of this variety seem to have a good field performance, much better than Ry_{adg} varieties. In conclusion, the application of MAS is severely limited by the low number of varieties that could amplify the markers, the capability of these varieties to flower and produce seeds and the agronomic performance of the progenies obtained.

Another reason for the limited number of published reports about the practical use of MAS could be that private seed companies usually do not give details of their methodology due to competition with other companies. In general, the problem of publishing extends also to the scientific community. New QTLs or *R* genes are often reported in scientific journals, but reconfirmation of these QTLs and *R* genes in other germplasm and identification of more useful markers are usually not considered novel enough to justify new publication. This is exactly the information needed for MAS application.

Finally, the low impact of MAS could also be due to the fact that although DNA markers were first developed in the late 1980s, user friendly ones such as SSRs were not developed until the late 1990s. With the advent of third-generation marker technologies such as the single-nucleotide polymorphisms, the power and efficiency of genotyping are expected to improve in the coming decades, and the promise of MAS for improving polygenic traits in a quick time-frame and a cost-effective manner could be a reality. However, it is also worth noting that large initial capital investments are required for the acquisition of this third-generation equipment, maintenance or personnel training and that this can widen even more the gap between the scientific and breeding communities or among well-developed and developing countries, reinforced by the secrecy or protection of intellectual property rights. Any SME or breeding institution should have to compare the costs of implementing the equipment required and the benefits of applying these technologies along the time. Moreover, to take advantage of these technologies, research teams, governmental agencies, commodity groups and the commercial sector will need to work together to insure a tangible impact on crop improvement.

Acknowledgements We would like to thank Dr. W.S. De Jong who kindly provided us the protocol of TG689 marker and Dr. Ana Carrasco who started to apply the molecular markers in Appacale.

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