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Molecular markers for plant genetics and breeding

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Abstract. The development of molecular markers has been a major step forward in understanding the genetic basis of phenotypic diversity, and in measuring the amount and organization of genotypic polymorphisms in wild and cultivated plant species. The progress in molecular technologies of the last 50 years has lead from a few isozyme markers to a virtually unlimited number of DNA-based markers that are highly polymorphic, codominant, ubiquitous in the genome, and can be obtained by relatively cheap and simple technologies. With markers it has been possible to introduce linkage as a new dimension in genetic analysis, allowing map construction, dissection of quantitative traits, association genetics, positional cloning of genes of interest and the study of genome evolution based on the comparison of the genome positions of homologous markers. These developments have led to multiple applications for plant breeding, including cultivar fingerprinting, major gene or QTL (quantitative trait loci) assisted selection, whole-genome selection in backcross programs, and genomic selection, which have been adopted by most breeders as tools to enhance the efficiency of conventional methods of plant improvement. This paper summarizes the research conducted in Catalonia that has been pioneering in this area at the international level, particularly for horticultural crops.

Resum. Els marcadors moleculars han permès un dels majors avenços recents en la comprensió de la base genètica de la diversitat fenotípica i la mesura de la variació genètica en plantes silvestres i cultivades. En els darrers 50 anys hem passat d'unes desenes d'isoenzims a un nombre virtualment il·limitat de marcadors d'ADN altament polimòrfics, codominants, presents a tot arreu del genoma i que poden ser obtinguts amb tecnologies senzilles i barates. Els marcadors han introduït el lligament com una nova dimensió en l'anàlisi genètica, permetent la construcció de mapes, la dissecció de caràcters quantitatius, la genètica d'associació, el clonatge posicional de gens d'interès i l'estudi de l'evolució dels genomes basada en la comparació de mapes. Els marcadors han estat també útils per la millora genètica, permetent la identificació molecular de genotips, la selecció assistida amb marcadors de gens majors i QTLs (loci de caràcters quantitatius), la selecció de tot el genoma en programes de retroencreuament i la selecció genòmica, que han estat adoptats com eines que augmenten l'eficiència dels mètodes convencionals. Aquest article resumeix la recerca realitzada a Catalunya en aquest àmbit que ha estat pionera a nivell internacional, en particular als fruiters i hortalisses.

Keywords: molecular markers, genetic variability, marker-assisted selection, plant improvement

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The evolution of molecular markers: from isozymes to next generation sequencing-based markers

After the rediscovery of Mendel's laws at the beginning of the 20th century, models of genetics, evolution and population genetics drawn up by pioneers such as R. Fisher, J.B.S. Haldane and S. Wright were essentially theoretical and their experimental validation had two major drawbacks: the observed variability was generally determined by dominant genes, and including linkage in the models was difficult. Only two phenotypes can be observed for a

single dominant *A/a* gene: the recessive class *aa*, and the dominant class *A-* (including the indistinguishable *AA* and *Aa* classes). This precludes a proper analysis of the adjustment of its segregation to a Hardy-Weinberg equilibrium or its departures caused by mutation, selection, migration, inbreeding or random drift. With regard to the second drawback, linkage, not intuited by Mendel, was discovered relatively early in the 20th century (T.H. Morgan and A.H. Sturtevant) and integrated in population genetics models although essentially as two or three-locus interactions. Linkage analysis was difficult because of the dominance of the genes available, and the fact that they were often mutants, natural or induced, where the aa genotype frequently had a deleterious effect on the phenotype. Working with many of these genes – also called major genes or morphological markers - was extremely laborious as they rarely segregated in natural populations, often requiring the complex and time consuming elaboration of plant stocks adequate to study their cosegregation and linkage. In short, the construction of linkage maps was an endeavor that required so much investment and time that it was restricted in plants to a few model crops: wheat, pea, maize, barley, rice and tomato.

The first markers that represented a real change were isozymes, variants of specific enzymes that retained their activity but had a slightly different amino acid composition that modified their shape or electric charge. They could be separated by electrophoresis and their position identified as bands with histochemical stains. While they were discovered at the end of the 1950s, their use in plant genetics became significant 15 years after, increasing until the 1980s, when they began to be substituted by DNA markers. Enzymes with one or a few copies in the genome were ideal, as they produced few bands of easy genetic interpretation, each corresponding to a different allele (allozyme) of one enzyme-coding gene (isozyme). Their advantage was that it was possible to access a set of variable, codominant genes that did not usually interact with each other or with the environment, and that allowed the variability between different populations and species to be compared. Their main disadvantage was their low number: a few tens per species.

In spite of this major limitation, isozymes opened up a new era in plant genetics and breeding. Using them made it possible to propose new goals in essentially two distinct domains: the analysis of genetic variability, and the use of markers in connection with linkage, i.e. to monitor changes occurring in neighboring genes - or by extension at the whole genome level - and to study evolution of the entire genome. Most of the new uses of markers for plant genetics and breeding were conceived and developed within a single decade (Tanksley and Orton, 1982), because the theoretical problems were well known and only required an adequate tool for their study. The genotypes of sets of isozymes in wild or cultivated populations provided extremely useful information on their level of variability and their history, and predicted their evolution under external constraints, including the evolution of breeding populations under selection. They also permitted a first comparison of the levels of variability of different species, established the importance of the mating system in the amount and distribution of variability across populations (Gottlieb 1981), and provided an elegant approach to identify species arising from recent polyploidy events (Gottlieb 1982).

Many applications of isozymes emerged: the identification of cultivars or genotypes, important for protection of breeder's rights or as quality control of propagating materials; tests for F1 seed purity, and evaluation of the parentage of individuals of unknown pedigree (Tanksley and Orton 1982). Other applications involved the use of isozymes linked to major genes of interest (marker-assisted selection) for earlier or more efficient selection, and the use of markers for rapid recovery of the recurrent genome in backcross programs (Tanksley et al. 1981). The dissection of quantitative characters in their component QTLs (quantitative trait loci) was first proposed at this time (Tanksley et al. 1982). Isozyme linkage groups in different species also suggested the conservation of genomic regions in broad evolutionary transects, which was the beginnings of comparative mapping (Tanksley and Orton 1982).

The first marker type allowing a direct analysis of DNA variation was RFLP (restriction fragment length polymorphism) (Botstein et al. 1980). Codominant markers of high quality could be produced, with the advantages over isozymes in that their number was virtually unlimited, and RFLPs identified variation in nearly every genome region, coding or noncoding. RFLPs are produced by digesting the total DNA of an individual with a restriction enzyme, separating the resulting fragments by electrophoresis, and detecting the positions of specific fragments using a short radioactively-labeled DNA probe. The RFLP method is time-consuming and expensive, but allowed the construction of the first complete (saturated) maps of many species, with tomato (Bernatzky et al. 1986) being the first in a long series of maps. For the first time it was possible to use genetic strategies requiring whole genome coverage,

allowing full realization of what could only be sketched with isozymes, such as QTL analysis (Paterson et al. 1989) and comparative mapping (Ahn and Tanksley, 1993). The abundance of markers also made it possible to find them where needed, particularly in the neighborhood of genes of interest, resulting in a generalized use of marker-assisted selection (MAS), and for whole-genome selection to accelerate backcross breeding programs (Tanksley et al. 1989). New avenues were also opened, with the possibility of cloning genes by successive identification of markers that were close to their position, with the help of large-fragment DNA yeast and bacterial artificial chromosome libraries (YACs and BACs).

The decade of the 1990s witnessed the development of many other kinds of markers that progressively substituted RFLPs, maintaining the high quality and number, with improvements in method simplicity and costs. Most were based on the polymerase chain reaction (PCR) along with sequencing technologies, especially Sanger sequencing using capillary electrophoresis, with markers more targeted to specific sequences or genome regions. Currently there are two main sources of markers based on the two major sources of DNA variability: SNPs (single-nucleotide polymorphisms) that detect single nucleotide substitutions, and SSRs (simple-sequence repeats), that use insertion/deletion polymorphisms in the widespread microsatellite sequences. New genetic analysis strategies were developed when the cost of genotyping large numbers of markers became reasonable. Bulked segregant analysis, a powerful approach to find markers close to specific genes of interest, was developed early on (Michelmore et al. 1991), and has been useful for finding markers for MAS or as a first step towards map-based cloning. Another key strategy was genome-wide association analysis, offering a wider range of variability for inheritance studies and allowing more precise mapping of genes/QTLs than with conventional biparental populations. Genotype-phenotype relationships can be predicted using a genotypically well characterized subset of individuals (the training population) from where a model of phenotypic prediction is tested and applied to a broader sample of individuals. This is the basis of genomic selection that has proved valuable in animal breeding, and promises to also be useful in plant species, particularly those with long-generation times such as fruit and forest trees.

The first genome sequenced in plants was that of Arabidopsis in 2000, but with the advent of next generation sequencing devices, this has become relatively affordable and genomes of most of the important crop species are now sequenced or will be in the near future. Whole genome sequences mean a simplification of genetic analysis as they provide a template with which low coverage random sequences (resequences) of one or a set of genotypes can be aligned, allowing the detection of often millions of markers (SNPs and indels). Having large sets of markers facilitates the saturation of specific map regions and the fine mapping of target genes and QTLs. The whole set of genes of a species are located in the genome, making it very simple to associate their positions with that of specific major genes or QTLs already described. Sets of markers with coverage of the whole genome can be designed for any application to breeding: fingerprinting, linkage map construction, association analysis and genomic selection, among other uses. With resequencing, specific genome regions containing genes of interest can be explored, and their genetic polymorphism analyzed in individuals with different phenotypes, allowing strong genotype-phenotype inferences.

Marker analysis research in Catalonia

Research at IRTA on horticultural crops

This began in 1982 at the Cabrils Center of SIA (Servei d'Investigació Agraria) with the research facilities transferred to the Government of Catalonia from the INIA (Instituto Nacional de Investigaciones Agrarias). In 1985, IRTA (Institut de Recerca i Tecnologia Agroalimentàries) was created, that included the SIA and other agricultural research groups in Catalonia. The initial research objectives were to establish a laboratory adequate for the work with isozymes, and to use these markers to understand genetic variability and its applications in breeding programs at IRTA or other public or private parties. Recently (2011), researchers in the fields of genetics and genomics moved to CRAG (Centre de Recerca en Agrigenòmica), a consortium of IRTA, CSIC (the Spanish Consejo Superior de Investigaciones Científicas) and the two main Universities of Barcelona (UB and UAB) at the UAB Campus in Bellaterra.

In the initial stages, peach, almond, hazelnut, walnut, carnation and carob were the target species, and we studied isozyme variability, inheritance and linkage. These data were useful for cultivar fingerprinting, of interest for quality control by nurserymen, and for identity testing for official organizations responsible for granting breeder's rights. In collaboration with a seed company, we studied the use of isozymes for seed purity testing in a set of vegetable crops (tomato, pepper, melon, cucumber and eggplant). In all,

isozymes were reliable and useful, but their use was highly dependent on the level of genetic variability of the species, given the limited number of marker loci available. For species with low variability, such as tomato, pepper and peach, the extent of the application was narrow, as the ability of the markers to discriminate was also low.

The situation changed with the development of PCR-based markers, starting from 1990. The first of these, known as RAPDs (random amplified polymorphic DNA), had various quality problems (dominance and low repeatability), but they were so abundant that it was often possible to find a sufficiently reproducible polymorphism for certain simple applications. This was a solution for the F1 seed purity test analysis, where one or two polymorphic markers between the two inbred lines of a hybrid were sufficient for establishing a robust and efficient test. These results were rapidly adopted by a collaborating seed company: the DNA test reliably confirmed the quality of F1 hybrid seed lots from different origins within a short period of time. The alternative was a time consuming and error-prone grow-out test, which involved the phenotypic identification of sibs (usually selfing progeny of the female inbred) from a sample of plants of each lot grown in the greenhouse. Since then, most breeding companies have used DNA markers for seed purity tests, a major quality procedure.

At that moment, RFLPs were the appropriate markers for constructing linkage maps, and we used these and isozymes to produce a saturated map for almond (Viruel et al. 1995), which was the first to be published and the most complete in stone fruit (*Prunus*). Between 1992 and 1996 we were granted a European project to construct maps for peach, cherry, almond and plum that I coordinated and which included French, Italian, British and Spanish partners. The approach was to elaborate a densely populated, high quality map for peach, and then construct maps of lower density in several populations of the other species using selected markers from the peach map. Given the low genetic variability of peach, an interspecific F2 almond x peach was chosen as the mapping population, which insured sufficient population and map coverage. This map (Joobeur et al. 1998) was adopted by the *Prunus* scientific community as the reference for the genus and was used to establish the chromosome terminology and orientation. There was similar development in melon, in which we strongly invested as it was high priority for one of our industrial partners. The first melon map was developed with RFLPs (Oliver et al. 2001), in the F2 from the cross between a line of the major cultivar produced in Spain (Pinyonet Piel de Sapo) and a Korean cultivar selected to be one of the most genetically distant cultivated melon accessions based on marker data.

We developed collections of the new SSR markers for our species of interest, mainly peach and other stone fruit, melon and strawberry, the latter a species with a complex octoploid genome for which we had a contract with a breeding company. SSRs were used to further saturate our reference maps, at the same time providing adequate markers to other research groups for the construction of maps in different populations and the study of other characters. Using maps from *Prunus* crops we found that all shared essentially the same genome and we were able to identify the positions of many major genes mapped in different populations from these species in a unique reference map (Dirlewanger et al. 2004). A method for mapping any marker using a reduced sample of plants (8) from this map was developed (Howad et al. 2005) and used in many other crops. In melon, a gene responsible for resistance to melon necrotic spot virus was map-based cloned (Nieto et al. 2006). Markers closely linked to disease resistances or other traits were developed for use in plant breeding, in the vegetable and fruit species in which we worked, some of which developed ourselves in collaboration with our industrial partners, and others developed by third parties that we adapted to our needs and conditions. Most of these markers have been adopted by breeders to aid faster and more efficient selection.

SSRs were also useful to study the variability of crops and for fingerprinting due to their high polymorphism, and because they were mapped and could be selected to cover the whole genome at regular intervals. SSRs provided an insight to the evolution of peach after its domestication, at least five millennia ago in China. Genotypes of large collections of cultivars showed that the original Chinese landraces (Li et al. 2015) were much more variable than the occidental and oriental breeding materials due to the extent of erosion in the gene pool generated by inbreeding, selection and random drift. In the case of occidental cultivars, markers were also able to separate materials from the modern breeding programs and a group of traditional Spanish peach non-melting flesh genotypes: the latter were essentially homozygous due to the long periods when they were seed reproduced, being clonally reproduced by grafting only in their most recent history.

An important outcome of these variability analyses was that they revealed the enormous power of SSRs for genotype fingerprinting. In 1996 a Spanish peach breeding company proposed we develop marker tests for charac-

terizing their varieties to use in the protection of breeder's rights. Later, with GESLIVE (Gestión de Licencias Vegetales) - a company created by the Spanish breeders association (ANOVE) - we elaborated a database with the SSR genotypes of the varieties owned by its associates, particularly of fruit, berry and cut flower crops. This database, which was produced with leaf materials from the official repositories of these species, contained the standards with which we could compare any sample sent for analysis by GESLIVE. DNA fingerprinting data have been accepted in court as strong evidence of identity between a known variety and material suspected of having been reproduced without license. The alternative in the past were field trials where the cultivars were compared by their phenotype, a long and expensive operation that made the defense of breeder's rights impractical. In peach, where isozyme variability was only capable of separating a few classes based on marker genotype, only 16 selected SSRs were able to identify virtually all materials. Individuals that were identical with markers were likely to be sports or duplications. The IRTAgen service, was created in 1999 to provide results from fingerprinting or other applications related with MAS to individuals, companies or the public administration. IRTAgen is still operative and has provided many thousands of analyses in almost every crop that is important in Spain. A change from SSRs to SNPs is currently underway to provide more reliable and cheaper tests for its clients.

One of the key elements for analyzing the relationship between genotype and phenotype is the generation of the appropriate tools to maximize its efficiency. We have developed many progenies for map construction and major gene or QTL analysis, with the most efficient being the Near-Isogenic line (NIL) collections. These consist of a set of plants with the genetic background of a certain elite genotype, each with a single DNA fragment from an exotic genotype. The sum of the exotic fragments of a NIL collection covers the whole genome of the exotic parent. Each NIL is an inbred line that can be reproduced by selfing, and any phenotypic difference when compared to the elite genotype can be attributed to the exotic DNA fragment. NILs are especially interesting for analyzing complex quantitative characters, allowing the exploration of the variability brought by exotic germplasm or wild relatives, detecting more QTLs than other classical populations - in part because they can be identified in the homogeneous background of the elite genotype. This allows the Mendelization of these QTLs, providing materials to study QTL interaction (by crossing two NILs with two different QTLs for a given character), and being a first step towards map-based cloning. The first NIL population we constructed was using the parents of the melon map, with 'Piel de Sapo' as the recurrent genotype (Eduardo et al. 2005). This population has been extensively used for the analysis of a diverse set of characters, including those related with fruit shape and weight, a quantitative resistance to cucumber mosaic virus, and to unravel the complexity of the climacteric vs. non climacteric ripening process in melon. Another NIL collection has been developed for diploid strawberry (*Fragaria vesca*), using the cultivar 'Reine des Vallées' as recurrent parent and an accession of F. *bucharica* as exotic donor (Urrutia et al. 2015a). With this collection it has been possible to find many major genes involved in different morphological and reproductive traits, and it is especially useful for the analysis of metabolites involved in fruit taste and aroma (Urrutia et al. 2015b).

Breeding in perennial species, such as fruit or forest trees, is limited by their long intergeneration periods, such that conventional breeding schemes are slow and inefficient. In peach this is a limiting factor, as the species is highly monomorphic, so hinders the enrichment of its genome with new genes from exotic materials or wild relatives. In fact, none have been introduced in a commercial cultivar while genes for disease resistance or longer postharvest life are in great demand. We proposed a new marker-based breeding scheme, marker-assisted introgression (MAI), to transfer one specific chromosome fragment from an exotic donor into an elite peach germplasm in only two backcross generations. In this method, markers are used to select individuals with a few introgressions in a large backcross one (BC1) progeny, and those with only one introgression in BC2 detected. A small collection of BC1 lines with a few introgressions allows a first survey of the new variability provided by the donor parent, with the detection of some of the major genes or QTLs that are dominant for the donor parent allele or additive. We have shown that the process works correctly in the offspring of almond × peach crosses in only eight years (Serra et al. 2016). This has been also useful to generate a collection of NILs of almond fragments in the peach background that we expect to share with the scientific community in the next few years.

We had an active role in sequencing full genomes for the species in which we are primarily interested, being involved in the creation of the sequencing consortia and participating directly in generation of the sequence, as was the case for strawberry (Shulaev et al. 2011) and peach (Verde et al. 2013). Given that three of the major genomes of the

Rosaceae (apple, peach and strawberry) were sequenced in a short time, it was possible to compare their genomes and infer their evolution from a common ancestral genome (Illa et al., 2011). The founder members of CRAG (CSIC and IRTA) lead the project for melon, along with private breeding companies, and public, Spanish and Autonomic, support. Using the shotgun approach with 454 sequencing technology, the 450 Mb genome was assembled and a set of more than 37,000 protein-coding genes annotated (Garcia-Mas et al. 2012). We have also been involved in the sequencing of the bean genome (Vlasova et al. 2016), and are currently in the consortia that are sequencing the genomes of octoploid strawberry and almond.

Research on molecular markers in cereals at IRTA, University of Lleida (UdL) and University of Barcelona (UB)

Breeding programs on durum wheat, barley, bread wheat and triticale were established in Catalonia at the beginning of the 1980's. Research on these species, particularly bread wheat and barley, was done in collaboration between IRTA and the UdL, with occasional participation of the UB. Markers were identified in these species as useful tools for germplasm management and variability analysis (Royo et al. 2010; Soriano et al. 2016), and to understand crop evolution (Molina-Cano et al. 1999; Igartua et al. 2015) with the aim of identifying useful variability for breeding. Additionally, markers have been used for the genetic analysis of complex characters (Giraldo et al. 2016), particularly those related with drought resistance (Comadran et al. 2008; Maccaferri et al. 2011), the identification of chromosomal rearrangements (Farré et al. 2012), and the location of important genes such as pest resistance (Martín-Sánchez et al. 2003). In spite of their high economic importance and the enormous international research that makes this group of species one of the best characterized at the genetic level, the genomes of cereals have been among the last to be sequenced due to their large size, so requiring massive economic and technological effort. Once this important development is complete, coupled with the tools adequate for whole genome analysis at affordable prices, a bright future for marker-based strategies to foster the improvement of this important group of crops is expected.

Research on molecular markers at the Polytechnic University of Barcelona (UPC)

A research line of the Department of Agrifood Engineering and Biotechnology at UPC is the collection, conservation, genetic analysis and sensory characterization of germplasm of locally important landraces and cultivars of vegetables. This research, partly supported by the Miquel Agustí Foundation (www.fundaciomiquelagusti.com), has been focused on beans, tomato, cabbage and onion. Markers have been used to genetically characterize these materials (Sánchez et al. 2007; Casals et al. 2011; Simó et al. 2014) and to study the inheritance of characters of interest for germplasm conservation (Casañas et al. 2013).

Conclusion

More than 40 years after first being used in plant genetics, molecular markers have become a necessary tool for genetic analysis, being essential for understanding the inheritance of complex characters, identifying genes responsible for these characters and in the search for novel variation. Their use is widespread in plant improvement programs where they have been incorporated in the three essential phases of the classical breeding methods: identification of adequate parents for crosses, selection in segregating progenies, and quality control of seeds or plants. The role of markers as predictors of the phenotype has been enhanced by the use of whole-genome approaches such as genomic selection, where important gains are expected in the years to come. Catalan groups have pioneered some of the research in molecular markers internationally, particularly in fruit trees and vegetable crops, where they have built strong and lasting collaborations with the private sector. This research is currently very active and, provided that public support is adequate, promises to continue producing good results and scientific leadership over the next few decades.

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