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Short Commentary Open Access

Use of Molecular Markers to Improve the Agro-Industrial Productivity in the North West of Argentina

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Genetic improvement of crop plants through conventional breeding has been the cornerstone to improve global agricultural production. Nowadays, different molecular biology techniques have become available to supplement and assist conventional breeding, among them the utilization of molecular markers. The generation of molecular, or DNA, markers are based on two basic molecular biology methods in order to be able to detect polymorphism: Southern blotting, a nucleic acid hybridization technique [1], and the amplification *in vitro* of specific DNA segments by the Polymerase Chain Reaction (PCR) technique [2]. By using these two methods and several variations of the basic technologies, many different kind of molecular markers have been developed during the last decades.

It is important to note that for a molecular marker to be efficient in crop breeding it should meet most of the following criteria [3]: high level of polymorphism; even distribution across the whole genome (not clustered in certain regions); co-dominance (or capacity to identify at heterozygous); clear distinct allelic features; single copy; low cost to use (or cost-efficient marker development and genotyping); easy assay/detection and automation; high availability (un-restricted use) and suitability to be duplicated/multiplexed (so that the data can be accumulated and shared between laboratories); genome-specific in nature (especially with polyploids) and have no detrimental effect on phenotype. However, the choice of the best marker system depends on many other factors as well including crop genetics and available resources (time, reagents, equipments, etc).

In Argentina, among the many public institutes and private companies working in plant breeding, the “Estación Experimental Agro industrial Obispo Colombres (EEAOC)”, founded in 1909 and located in the Province of Tucumán, occupies a preponderant place (http://www.eeaoc.org.ar/) with successful ongoing breeding programs in sugarcane, soybean, common bean and citrus. EEAOC is a Provincial Institute managed by the producers themselves and financed directly with resources from the production, which provides technology development and solutions, which contributes to improved productivity and sustainability of the agro industrial sector of the North West of Argentina. In 2012, the “Instituto de Tecnología Agroindustrial del Noroeste Argentino (ITANOA)”, a bioeconomy research institute of dual dependence between EEAOC and Consejo Nacional de Investigaciones Científicas y Técnicas” (CONICET) was founded as an institutional innovation (http://www.tucuman-conicet.gov.ar/Secciones.php?IdSeccion=51). The main objective of this new Institute is to contribute to a sustainable development in social, environmental and economic terms through technology transfer in order to improve productivity, health and industrial processing of crops and plant biomass in the region. The overall idea is to generate, integrate and coordinate a social-economic development with agro-industrial production and environmental protection, based on creativity and knowledge. EEAOC-CONICET-ITANOA is working on the economically most important crops of the North West of Argentina, which includes sugarcane, soybean, lemon and strawberry.

Sugarcane production in Argentina is the oldest agro-industrial activity in the country, starting in the late 19th century. Sugar production is historically concentrated to the Provinces in the North West of Argentina (Tucumán, Salta and Jujuy) although there is a small sugar development in the North East of the country [4]. Annually about 2 million tons of sugar are produced from around 350,000 hectares cultivated. The Province of Tucumán is the main sugar producer in the country with approximately 66% of the total production from approximately 265,000 hectares, representing about 45% of the total agricultural area of the Province [5]. In addition to its economic importance, the sugar industry plays an important social significance for Tucumán since there are around 5,500 farmers and it generates more than 20,000 jobs.

Commercial sugarcane varieties all belong to Saccharum genus and are inter-specific complex artificial hybrids characterized by a high degree of polyploidy and frequent aneuploidy [6]. These characteristics and the cytogenetic complexity of sugarcane cultivars, involving varying chromosome sets and complex recombinational events, imposes difficulties in accomplish effective breeding programs [7]. In Argentina, the first sugarcane breeding program was formally established in 1968 by the EEAOC. Completing a sugarcane breeding cycle takes at least 11 years, starting with a crossing between two elite clones, evaluating the progeny to identify true hybrids, several stages of testing and clonal selection, and finally ending with a new variety release. In order to broaden the genetic base of commercial varieties of sugarcane, it is important to identify more genetically diverse parents to be used in breeding programs [8]. For that reason and for protecting new sugarcane varieties and intellectual property rights, an accurate varietal identification is essential [9]. In addition, the knowledge of the genetic diversity in sugarcane will provide useful information concerning the genotype value to breeders and will contribute to the improved use and conservation of genetic resources.

Molecular markers are powerful tools to estimate genetic diversity and to generate information to better understand the complex genetics of sugarcane as they are accurate, abundant and not affected by the environment [10]. Random Amplified Polymorphic DNA (RAPD) was the first molecular marker technique employed at EEAOC to characterize sugarcane genotypes [11]. This relatively simple DNA amplification technique allows for application without the need to know the sequence of the DNA to be amplified in advance. The technique generated useful information in sugarcane and allowed for the identification of related genotypes; however the short length of the primers implies that DNA hybridization are performed at a

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relatively low temperature, which increase the likelihood of a non-specific alignment and generation of ambiguous information by unspecific amplification and poor reproducibility [12]. To solve this problem, more recently, Amplified Fragment Length Polymorphisms (AFLP) and Simple Sequence Repeats (SSR) markers were used to characterize the sugarcane genotypes mostly used as parents in the sugarcane breeding program at EEAOC [13,14]. As sugarcane has a very large and complex genome a vast number of markers, such as those generated by AFLP techniques [15], are necessary to determine the genetic diversity [16]. An advantage of using AFLP markers is that it allows for simultaneous screening of many different genome regions distributed randomly throughout the genome [17]. In our study we found a high number of AFLP bands but a very limited polymorphism (19.40%) that reflects a low genetic diversity in the breeding germplasm evaluated (Jaccard coefficient mean value: 0.96). However, the AFLP technique still provides a useful alternative for diversity estimation as well as for genotype identification because the 16 primer combinations tested were enough to differentiate between all sugarcane genotypes characterized. Another powerful technology for monitoring genetic diversity and varietal identification is SSR because of their abundance, sensitivity and high accuracy in detecting polymorphism even between very closely-related genotypes [18]. It must be highlighted that although SSRs are classified as co-dominant type markers, they have been treated as dominant markers when analyzing the highly complex genome of Saccharum. In our work, SSR markers had a mean genetic diversity value lower than AFLP (0.84) but reflected genetic relations more accurately than AFLP. However, the correlation between AFLP and SSR dendrograms (0.58; $p<0.05$) indicates that there is a concordance between these two techniques, despite the difference in topology of the dendrograms. In order to obtain a more accurate estimation of the genetic diversity we combined the use of both molecular markers with morphological traits. The benefits of the genetic knowledge obtained by the implementation of molecular markers together with morphological traits was extremely useful not only to identify parental lines for favorable combinations in breeding for important traits but also to broaden the general genetic basis of the local germplasm [14]. In addition, the genetic variability established for the EEAOC germplasm by our study will be an important help when selecting parental crosses for genetic mapping and discovery of potential quantitative trait loci.

It must be pointed out that some authors have suggested that the estimation of genetic diversity through molecular markers for breeding purposes should be based primarily on functionally characterized genes or genes of interest, since they reflect functional polymorphisms [19]. Variability of expressed or regulatory sequences might reflect a selection pressure for these regions, which would be different for each gene. Therefore, the variation detected by functional markers randomly distributed across the genome may detect variability more useful for breeders, since reflect genetic variations of direct interest. There are several molecular techniques to detect genetic variation in coding regions. One such marker is the Target Region Amplified Polymorphism (TRAP), which is a PCR-based marker that is designed to detect intragenic polymorphisms. These markers are generated with a fixed sense primer of about 18 nucleotides, designed from a gene or EST (Expressed Sequence Tag) and an antisense arbitrary primer of the same approximate size, designed with an AT- or a GC- motif, in order to direct them to hybridize with introns or exons, respectively [20]. These properties of TRAP markers make them an interesting tool to detect polymorphisms that can be useful for genetic improvement when analyzing gene-rich regions of a genome. This marker technique was tested under our laboratory conditions for ten sugarcane genotypes [21] and when TRAP and AFLP markers were compared, a very similar number of average bands were obtained (47 vs. 52). However, TRAP markers revealed a much higher polymorphism, 59.7%, as compared to the AFLP markers which only showed a 34.43%. These results indicated that TRAP markers are more effective than AFLP when analyzing the sugarcane genome, as it is easier, faster and more economic than applying AFLP markers and more efficient as seen by the number of polymorphic loci obtained for each reaction [21]. It must be highlighted that in most cases a combination of different marker techniques is recommendable for genetic diversity studies since they could reflect different aspects of the genome studied as well as providing more accurate information. Nevertheless, TRAP markers are routinely applied as a first approximation in genetic diversity studies in our laboratory.

As previously described the choice of marker system depends primarily on the purpose of the study. In that sense, SSR markers are ideal to determine self-pollination accurately and to identify true hybrid progeny, even at very early stages of a breeding program [22]. In a breeding program it is essential to assure the hybrid identity of the progeny obtained from the crosses, and SSR is routinely used to test the nature of the progeny obtained from the crosses in our sugarcane breeding program. In addition, these markers are important tools to confirm the success of an emasculation treatment for plants used in crossings [14,23].

When considering the complexity of the sugarcane genome and the amount of information generated in our laboratory for different purposes, the availability of an automated system for the rapid and efficient detection of molecular markers is essential. In that sense, the Licor 4300 DNA Analyser permits for real-time detection of molecular markers since it automates the entire process, from the electrophoresis step to the registration of presence/absence of markers in multiple samples. The combination of an infrared band detection system with the use of the SAGA™ software permits the automatic transfer of image data collected during electrophoresis, allowing for a comprehensive and highly reliable analysis of the molecular markers generated. It must be pointed out that specifically fluorescent reagents developed by manufacturers must be used to produce detectable bands by the laser system, increasing the cost of generating data. In order to optimize the use of the fluorescent reagent and lowering costs we have developed PCR reactions based on dCTP coupled to a fluorescent Cy5.5 (Cy5.5-dCTP; GE Healthcare Life Sciencies) for all markers used in our laboratory. It is important that the ratio of dCTP coupled to fluorescent/dCTP is optimized in each PCR reaction since the presence of several cytokines coupled to a fluorescent in an amplified product can cause inhibition of DNA polymerization. Today, in our lab, all molecular marker systems employed routinely have been optimized for their detection by the Licor 4300 DNA Analyser equipment [20].

Another important application of molecular markers in breeding programs is selecting genotypes with important agronomic traits at an early stage, which is an important help in order to reduce times and costs in the selection process. A good example of such an application is the case of sugarcane brown rust disease (*Puccinia melanocephala*). The most effective method to control this disease is the use of resistant commercial varieties and a recent study has reported that brown rust resistance in modern sugarcane cultivars relies essentially on the *Bru*1 gene [24] that presents complete linkage disequilibrium with two flanking molecular markers, R12H16 and 9O20-F4. These two markers are reliable and reproducible markers for the presence of *Bru*1, since their presence predicts a resistant behavior of any modern sugarcane cultivar. Whereas the absence of these markers in a resistant cultivar would indicate the absence of *Bru*1, suggesting an alternative source

of resistance [24]. The usefulness of these two molecular diagnostic markers in order to predict a rust resistant phenotype under natural high pressure inoculums conditions was analyzed by evaluating natural field infections of different sugarcane genotypes. Genotypes were evaluated for resistance or susceptibility to brown rust and subsequently screened for presence or absence of the two *Bru*1 diagnostic markers. Thirty eight percent of the genotypes were determined as resistant to brown rust but only 16.3 % of them were found to harbor the *Bru*1 gene as determined by the two markers. This result supports the idea that *Bru*1 diagnostic markers enable a rapid and reliable positive selection for brown rust resistance in sugarcane. Interestingly, our study revealed at least one additional source(s) of resistance within the germplasm used in the study. Very little genetic variability of rust resistance independent of *Bru*1 has been reported previously, and it is striking that this alternative genetic resource(s) found in our local germplasm constitutes the predominant one. Further research regarding the nature of this resistance is needed but there is no doubt that this finding should be helpful in order to expand the narrow genetic basis for brown rust resistance in sugarcane in the future [25].

Molecular markers associated with important agronomic traits could significantly reduce the time and cost involved in developing new varieties because they help in selecting the best parents and accelerating the rate of genetic gain in the breeding program. Association mapping has become widely used to identify DNA markers associated to important traits in many crops. In order to find valuable markers for the local breeding program, a Genome Wide Association Study (GWAS) was used to find markers associated with high biomass and sugar yield in a local sugarcane population [20]. In this study two distinct types of molecular markers were used: TRAP and Diversity Arrays Technology (DArT). The recently developed technology of DArT in sugarcane [26] has made it possible to conduct a genome-wide scan of this genetically complex crop, capturing genomic profiles with many thousands of polymorphic markers. Results from this study revealed numerous molecular markers significantly associated with both phenotyped characteristics. Our findings support previous results in other plant species that this approach allows for the identification of marker alleles responsible for variations in important quantitative traits, enabling these markers as a valuable tool to assist the improvement of productivity of sugarcane to better supply the sugar and biomass demand that has been projected for the upcoming decades [20].

As stated before, due to the extremely complex genome of sugarcane conventional breeding is slow and laborious. In addition, many traits of interest are not found in the cultivated germplasm or in closely related species, preventing the introgression through sexual crossing. Thus, genetic transformation is therefore an invaluable tool to overcome such limitations contributing directly to the introduction of novel and improved traits [27], which can generate increased yields, lower management and manufacturing costs and lead to a more sustainable crop production [28]. In a pioneer project EEAOC obtained a transgenic sugarcane plant of the local commercial variety RA 87-3, transformed with a genetic construct harboring the *epsps* gene responsible for conferring tolerance to the wide spectrum herbicide glyphosate and the *npt*II gene for kanamycin selection of transformed plant cells. In order to verify stable expression and enzymatic activity of the introduced genes, molecular and biochemical studies were conducted. Furthermore, genetic studies using molecular markers were performed to ensure that no genetic recombination through somaclonal variation had occurred, since it is a well-known fact that genetic transformation and *in vitro* regeneration processes can cause important genetic changes [29]. In summary, our results showed that the use of TRAP markers to genetically characterize promising transgenic lines is a rapid and recommendable first approach to identify transformed plants genetically close to their parental genotype. Furthermore, our studies indicate that TRAP markers are more adequate to detect genetic changes caused during the transformation process than other molecular markers techniques such as RAPD [30] and AFLP [31] which previously have been used in sugarcane where they failed to detect genomic differences between clones with visually distinct phenotypes. In addition Southern blot analysis was performed using two restriction enzymes to determine the number of transgene insertions. Both techniques are routinely applied in our lab to select for good genetic candidates at a very early stage of the transformation process.

As stated above, the most effective way to control brown rust and other sugarcane diseases is the use of resistant cultivars. For that reason, the local breeding program of the EEAOC is continuously working to release resistant varieties to the most important local diseases. However, other strategies to manage diseases in sugarcane are also important and EEAOC is, since 2001, conducting an ambitious seed cane sanitation project (Vitroplantas). On average, a total of 85,000 sugarcane seedlings representing all important commercial sugarcane varieties in Tucumán are produced annually through *in vitro* meristem cultures. All *in vitro* seedlings are rusticated in a special greenhouse with low light and high humidity and thereafter planted in a Basic nursery. All plants are conventionally propagated in two more stages in Registered and Certified Nurseries, before being distributed among sugarcane growers for commercial production. The idea behind this project is to guarantee seed canes of extremely high phytosanitary quality and genetic purity. The sanitation of the plant material is achieved through *in vitro* cultures of apical meristems from donor plants previously hydro-heat-treated and grown under natural light conditions in greenhouses with anti-aphid screens during a maximum of 3 years. This micropropagation technique is widely used for the elimination of viral systemic diseases. To guarantee that meristem donor plants and micropropagated seedlings are disease free, both types of plants are evaluated by different molecular diagnosis developed for each important disease. PCR protocols to detect the two bacterial diseases, ratoon stunting (*Leifsonia xyli* subsp. *xyli*) and leaf scald (*Xanthomonas albilineans*) and RT-PCR protocols to detect the two viruses Sugarcane mosaic virus (SCMV) and Sorghum mosaic virus (SrMV), causing the sugarcane mosaic disease [22] and the sugarcane yellow leaf virus (SCYLV) that causes sugarcane yellow leaf disease, are routinely applied. The incorporation of the diagnosis of the last virus was introduced routinely after confirmation of the presence of SCYLV in sugarcane producing areas in Argentina [32]. As a result of the introduction of the Vitroplantas project systemic disease incidences have significantly decreased in the sugarcane production fields of Tucumán [33,34]. As mentioned previously in the genetic transformation part, sugarcane is very prone to somaclonal variation when micropropagated *in vitro* [35]. It is therefore of uttermost importance to be able to detect genetic changes during the propagation process to avoid releases of genetically distinct plants, which would compromise the productivity. In order to secure genetic purity of all cane seed released through the Vitroplantas project, a molecular approach based on molecular markers has been developed enabling us to quantify and detect genetic variations in propagated material after it enters rustication in the greenhouse. In cases where genetic changes are detected all clones originating from genetically distinct plant are destroyed to avoid release of inadequate material [36]. In the early stages of this project, AFLP markers were applied to ensure that no genetic changes in propagated material had occur, but taking into account the above mentioned multiple advantages of

the TRAP technology, it has replaced AFLP analysis completely and is now routinely employed to genetically characterize propagated plant material. In regards of productivity, efficiency and safety, meristem micropropagated plants are quite advantageous as they are healthy high yielding plants that in the short term will replace old and/or infected materials. This state-of-the-art technology, which is relatively wide-spread in sugarcane producing countries, was developed by the EEAOC to obtain seed cane of the highest phytosanitary quality and genetic purity, and to offer them to local growers in order to improve productivity [37].

One of the most important factors that negatively affect sugarcane production is the presence of pathogens. As mentioned earlier the best way of handling diseases is the use of resistant cultivars, which together with other measures including careful planning of crop management practices (time of planting and harvesting) or in the specific case of some fungal diseases the application of fungicides, are all helpful tools for limiting the effects of diseases. Nevertheless, in all disease situations a good knowledge of the genetic diversity of the pathogens as well as its interaction with the cultivar, allows for a more efficient and sustainable disease management. Once again molecular markers are an invaluable tool to study the genetics of pathogens. Sugarcane mosaic (caused by SCMV and SrMV), is one of the most important viral diseases of sugarcane that is widely distributed in the world with different economic significance among regions [38]. In order to provide valuable information for the development of appropriate *in vitro* diagnostic tests as well as for determining mechanisms for increased disease resistance, we isolated and analyzed the genetic diversity of viruses associated with sugarcane mosaic in the North West of Argentina, particularly in the Province of Tucumán. In this study we found that although the RT-PCR-based RFLP method of CP genes proposed by [39] facilitated a rapid identification and discrimination of strains from unknown field isolates, this method is not completely reliable; as a single nucleotide mutation is sufficient for an isolate to lose a restriction site and hamper typing by this method [37,40]. The two most importantly fungal diseases in sugarcane are brown rust, caused by *Puccinia melanocephala* H. and P. Sydow, and orange rust, caused by *P. kuehnii* (Krüger) Butler. The importance of rust in sugarcane is highlighted by the fact that most breeding programs worldwide today constitute selection for resistance. It is important to notice that although brown rust was first detected in Argentina as early as in 1988 [41], very little genetic information of the pathogen is available. The lack of information regarding orange rust is even more pronounced as it was only very recently detected in Argentina [42]. Once again the direct genetic diversity assessment of the pathogen and indirectly the population structure and epidemiology, are essential to be able to design efficient management strategies. For those reasons, both pathogens are currently being characterized genetically by using AFLP markers in collaboration with the Cuban "Instituto de Investigaciones de la Caña de Azúcar" (INICA) [43].

Another disease with important dispersal that affects sugarcane production, and as a consequence the economy of the crop, in our region is red stripe caused by the bacteria *Acidovorax avenae*. Taking into account the importance of this disease in the region, several studies are being carried out by our Institute to better characterize the causal agent and estimate its genetic diversity by using molecular fingerprinting techniques. We are primarily conducting studies using the Repetitive Element Polymorphism-based polymerase chain reaction (rep-PCR) fingerprinting since it is a reliable and very efficient method to discriminate among closely related bacterial species by analyzing the distribution of repetitive DNA sequences [44]. This technique implemented in our lab to characterize important bacteria isolated from the intestine of *Diatraea saccharalis* larvae, had allowed to differentiate isolates and genotypes of *Klebsiella oxytoca*, *K. pneumoniae*, *K. variicola* and *Bacillus pumilus*, which showed the highest cellulolytic activity [45].

Another very significant crop in the North West of Argentina is soybean (*Glycine max* (L) Merr), the most important oilseed crop in the world. Soybean occupies a very important place in the economy of our country and in South America as a whole, where the three largest producers are Brazil, Argentina and Paraguay with 52, 41 and 4% of the regional production, respectively. In Tucumán, almost 300.000 hectares are planted with this crop and the breeding program of the EEAOC is continuously working to obtain new and better varieties adapted to local growth conditions in order to improve yields. Every breeding program basically requires the use of genetic variability as a starting point to produce combinations of genes that result in varieties with desired traits. The EEAOC genetic breeding program is of no difference and has a large germplasm bank from which crosses are designed annually. In order to be able to better design crosses, 112 soybean genotypes from the EEAOC germplasm were genetically characterized using 15 SSR primer pairs. Our results showed a similarity range from 0.11 to 1 (Jaccard´s coefficient), allowing of the differentiation of several advanced lines that could not be distinguished by conventional morphological and physiological descriptors [46]. It must be pointed out that as soybean is a diploid plant (2n=40) SSR markers were analyzed as co-dominant markers. In addition, the optimization of SSR in soybean allowed us to act, requested by a private company, as expert part in a litigation to identify soybean varieties of its property.

An important factor that affects soybean production is the Asian soybean rust (SBR) caused by the fungal pathogen *Phakopsora pachyrhizi* Syd. & Syd. This is one of the main diseases affecting soybean production and has been reported as one of the most economically important fungal pathogens worldwide. The presence of SBR in Argentina was first confirmed by molecular diagnosis studies conducted by researchers at the EEAOC [47]. In addition, to achieve the goal of determining the genetic diversity of this fungus in the region as part of a project to assist the soybean breeding program of the EEAOC to develop effective resistance breeding strategies, a method that combines a simple collection of urediniospore with a subsequent analysis by AFLP to examine genetic difference among samples of *P. pachyrhizi* was designed. The implementation of this novel methodology allowed determining the genetic diversity of the pathogen and will be useful when carrying out wider genetic diversity studies in the future [48].

Argentina is the world's largest producer of lemons, with 96% of the total national production concentrated in the Province of Tucumán, where 70% of the production is industrialized and the remaining 30% is sold as fresh fruits, mainly for export. The first application of molecular markers in this crop at the EEAOC was the use of SSR markers in order to correctly assure the hybrid nature of progeny obtained by the EEAOC rootstock breeding program [49]. Citrus production and marketing are greatly negatively affected by quarantine and emerging diseases. Among these, the Asian citrus canker, caused by *Xanthomonas citri* subsp. *citri* has an important economic impact on the citrus industry. To gain insight into the molecular epidemiology of canker disease, Xanthomonas isolates were collected from a range of Citrus species across different fields in Tucumán, and characterized by the method of rep-PCR. This analysis discriminated four distinct clusters, consistent with the four main Xanthomonas groups: *X. citri*, *X. aurantifolii*, *X. campestris* and *X. vesicatoria*, respectively. All collected isolates from trees showing disease symptoms were clearly distinguished among them and grouped with the *X. citri* control as

expected [50]. The interaction between *Citrus limon* and an isolate of *Xanthomonas citri* subsp. *citri* that presented a lower virulence in lemon, was characterized. Transcriptomic profiles were produced by using cDNA-AFLP technique and numerous fragments specifically induced or repressed by the isolates, were detected [51]. These analysis of the global expression pattern identified different classes of genes involved in plant–pathogen interactions. This work constituted the first approach in the study of specific genes that could be used in resistance breeding against the disease.

Another citrus disease responsible for significant economic losses worldwide, caused by the bacterium Candidatus *Liberibacter* spp. is huanglongbing (HLB). Today no effective disease control has been found, and the non-cultivability of the bacterium has severely hampered studies on the pathogen. For those reasons, we have developed for the first time a set of qPCR primers based on the conserved 16S rDNA gene, which specifically and simultaneously detects in a singleplex reaction, all three bacterial species associated with HLB. At the same time this technique allows differentiate *Ca. Liberibacter asiaticus* or *africanus* from *americanus* by their characteristic melting curves. The application of this fast, simple and efficient detection methodology could contribute to early pathogen detection, a crucial step in the development of preventive strategies aimed at avoiding the dissemination of this devastating disease in HLB-free areas [51].

Another important crop of the region where we have applied molecular markers is strawberry; Argentina is one of the four major producers in South America, with continuous production during the whole year. In addition to fruit production, Argentina is also providing ideal conditions for a competitive production of strawberry seedlings. In the Province of Tucumán both production of fruits and seedlings takes place [52]. Even though strawberry production is costly, this crop has experienced significant growth in the last decades and Tucuman has been positioned as the main producing province in Argentina of frozen fruits. In an attempt to genetically improve the productivity of this crop, during the 90s, the "Programa Nacional de Mejoramiento Genético de la Frutilla" (the Strawberry National Breeding Program) was founded and the program received funding from the National Research Council. Moreover, the program involved the establishment of an Active national Germplasm Bank of strawberry, with the aims of collecting, preserving, propagating and providing strawberry germplasm, both for breeding programs and for different public research projects. The preserved germplasm was botanically, genetically, molecularly, phytopathologically and agriculturally characterized [53]. Molecular markers were a newly developed tool that was useful in assisting the breeding program of this important crop for the region. As an example, RAPD markers were used to estimate the genetic diversity of the germplasm bank. Parents used in crossings were genotyped and to our surprise genetic variation among accessions of the same cultivar was detected, which is probably due to the accumulation of mutations and or genetic rearrangements during the vegetative propagation of the variety [54].

Strawberries are susceptible to a wide range of diseases caused by viruses, bacteria, fungi and nematodes that are fairly well characterized. Two of the most important fungal diseases affecting strawberry production are the grey mould caused by *Botrytis cinerea* [26] and anthracnose caused by fungi of the genera Colletotrichum [55]. Three species of Colletotrichum are known to be the causal agents of anthracnose in strawberry worldwide (*C. acutatum*, *C. fragariae* and *C. gloeosporioides*) and all three species were detected, with *C. acutatum* being the predominant species, in Argentina [56]. Different strategies were employed in order to identify molecular markers associated to resistance to anthracnose in strawberry. Bulked segregant analysis combined with AFLP was useful to identify polymorphisms linked to resistance in hybrids derived from a cross between a resistant cultivar and a susceptible one. Three polymorphic bands were detected only in resistant genotypes, including one that showed significant BLASTX hits to ADF proteins (Actin depolymerizing factors) from other plants, which are known to be implicated in plant defense against pathogenic fungi [57]. On the other hand, the differential gene expression between a susceptible and resistant genotype was analyzed by cDNA-AFLP technique. At least 60 different genes were found to be specifically expressed in the resistant genotype and interestingly some of them are associated with pathogen defense responses in plants [58].

On the other hand, the genetic diversity of all three causal agents was estimated by using ISSR markers (Racedo 2007). Furthermore, as part of the work we reported a new disease in strawberry plants caused by the fungus *Acremonium strictum*, which was detected by both the structure of conidiophores and the sequence spanning the internal transcribed spacers 1 and 2 of the nuclear ribosomal DNA [55]. It is worth mentioning that the correct identification of this causal agent allowed registering a patent of a bio-product whose active ingredient that induces the defense response in plants [59], was obtained from *A. strictum* [60].

In summary, we have employed the use of molecular markers in many different crop species and for different purposes. It is important to notice that almost all our actions are aimed to provide concrete solutions to the agro-industrial productive sector by providing better and more efficient plant germplasm and disease management solutions. The idea behind is to increase the economic, environmental and social sustainability of a sector which plays a decisive role for the developing of the North West of the country.

References

1. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503-517.
2. Mullis KB (1990) The unusual origin of the polymerase chain reaction. Sci Am 262: 56-61, 64-5.
3. Jiang GL (2013) Molecular markers and marker-assisted breeding in plants. In: Agricultural and biological sciences. Plant Breeding from Laboratories to Fields. Sven Bode Andersen, ISBN 978-953-51-1090-3. DOI: 10.5772/52583
4. Pérez D, Fandos C, Scandaliaris J, Mazzone L, Soria F, Scandaliaris P (2007) Estado actual y evolución de la productividad del cultivo de caña de azúcar en Tucumán y el noroeste argentino en el período 1990-2007.Publicación Especial 34 EEAOC.
5. Fandos C, Scandaliaris J, Scandaliaris P, Soria F, Carreras Baldrés J (2014) Area cosechable y producción de caña de azúcar y azúcar para la zafra 2014 en Tucumán. Reporte Agroindustrial N°94. Relevamiento satelital de cultivos en la provincia de Tucumán. Available in http://www.eeaoc.org.ar
6. Cordeiro GM, Taylor GO, Henry RJ (2000) Characterisation of microsatellite markers from sugarcane (*Saccharum* sp.), a highly polyploid species. Plant Sci 155: 161-168.
7. Vettore AL, da Silva FR, Kemper EL, Arruda P (2001) The Libraries that made SUCEST. Genet Molec Biol 24:1-7.
8. Salem KFM, El-Zanaty AM, Esmail RM (2008) Assessing wheat (*Triticum aestivum* L.) genetic diversity using morphological characters and microsatallite markers. World Journal of Agricultural Sciences 4: 538-544.
9. Wagih ME, Musa Y, Ala A (2004) Fundamental botanical and agronomical characterization of sugarcane cultivars for clonal identification and monitoring genetic variations. Sugar Tech 6: 127-140.
10. D'Hont A, Seshagiri Rao P, Alleyne S, Glaszmann JC, Feldmann P (1997) Application of molecular markers in sugarcane breeding. Proceedings West Indies sugar technologists 77-79.
11. Fontana PD, García MG, Cuenya MI, Chavanne E, Ontivero M, Díaz Ricci JC,

Castagnaro AP (2003) Utilidad de la técnica de RAPD de alta resolución para la caracterización molecular de dos genotipos emparentados de caña de azúcar (*Saccharum* spp). Avance Agroindustrial 24: 25-26.

12. Otero AA, Cruz M, Oyama K (1997). El uso de los RAPDs como marcadores moleculares en plantas. Boletín de la Sociedad Botánica de México 60: 85-117.
13. Perera MF (2011) Biotecnología en caña de azúcar: identificación varietal, estimación de la diversidad genética, diagnóstico molecular de patógenos y caracterización de virus relacionados con la enfermedad del mosaico. PhD Thesis. National University of Tucumán, Argentina.
14. Perera MF, Arias ME, Costilla D, Luque AC, García MB, Cuenya MI, Racedo J, Ostengo S, Filippone MP, Castagnaro AP (2012a). Evaluation of genetic diversity in sugarcane cultivars based on DNA markers and morphological traits". Euphytica 185: 491–510. DOI: 10.1007/s10681-012-0661- 9.
15. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, et al. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407-4414.
16. Lima ML, Garcia AA, Oliveira KM, Matsuoka S, Arizono H, et al. (2002) Analysis of genetic similarity detected by AFLP and coefficient of parentage among genotypes of sugar cane (*Saccharum* spp.) Theor Appl Genet 104: 30-38.
17. Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. Trends Ecol Evol 14: 389-394.
18. Powell W, Machray G, Provan J (1996) Polymorphism revealed by simple sequence repeats. Trends Plant Sci 1: 215-222.
19. Alwala S, Suman A, Arro JA, Veremis JC, Kimberg CA (2006) Target región amplification polymorphism for assessing genetic diversity in sugarcane germplasm collections. Crop Science 46: 448–449.
20. Li G, Quiros CF (2001) Sequence related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in Brassica. Theoretical and Applied Genetics 103: 455-461.
21. Racedo J (2014) Aproximaciones biotecnológicas aplicadas al mejoramiento de caña de azúcar para la obtención de variedades diferenciadas en su potencial industrial: azucareras y alcoholeras. PhD Thesis. National University of Tucumán, Argentina.
22. Zhang MQ, Zheng XF, Yu AL, Xu JS, Zheng H (2004) Molecular marker application in sugarcane. Sugar Tech 6: 251-259.
23. Perera MF, García MB, Díaz Romero C, Cuenya MI, Filippone MP, Castagnaro AP (2012c) Biparental crosses confirmed by SSR with mendelian inheritance in sugarcane breeding. Revista Agroindustrial y Agrícola de Tucumán 89: 1-7.
24. Costet L, Le Cunff L, Royaert S, Raboin LM, Hervouet C, et al. (2012) Haplotype structure around Bru1 reveals a narrow genetic basis for brown rust resistance in modern sugarcane cultivars. Theor Appl Genet 125: 825-836.
25. Racedo J, Perera MF, Bertani R, Funes C, González V, Cuenya MI, D´Hont A, Welin B, Castagnaro AP (2013a) Alternative sources of resistance to sugarcane brown rust disease. Euphytica 191: 429-436. doi: 10.1007/s10681-013-0905-3
26. Heller-Uszynska K, Uszynski G, Huttner E, Evers M, Carlig J, Caig V, Aitken K, Jackson P, Piperidis G, Cox M, Gilmour R, D'Hont A, Butterfield M, Glaszmann JC, Kilian A (2011) Diversity Arrays Technology effectively reveals DNA polymorphism in a large and complex genome of sugarcane. Mol. Breed 28: 37-55.
27. Vellicce G, Díaz Ricci JC, Hernández García L, Castagnaro AP (2006) Enhanced resistance to Botrytis cinerea mediated by the transgenic expression of the chitinase gene ch5B in strawberry. Transgenic Research 15: 57-68.
28. Noguera AS, Paz N del V, Díaz ME, Perera MF, Díaz Romero C, García MB, Filippone MP., Welin B, Cuenya MI, Digonzelli P, Castagnaro AP (2015) Production of healthy seed cane in Tucumán, Argentina. International Sugar Journal 112-116.
29. Gilbert R, Glynn N, Comstock J, Davis M (2009) Agronomic performance and genetic characterization of sugarcane transformed for resistance to sugarcane yellow leaf virus. Field crops Res 111: 39-46.
30. Taylor PWJ, Fraser TA, Ko H-L, Henry RJ (1995) RAPD analysis of sugarcane during tissue culture. In: Terzi M, Cella R, Falavigna A (eds) Current issues in plant molecular and cellular biology. Kluwer Academic Int., Dordrecht 241-246.
31. Joyce P, Hermann S, O'Connell A, Dinh Q, Shumbe L, Lakshmanan P (2014) Field performance of transgenic sugarcane produced using Agrobacterium and biolistics methods. Plant Biotechnol J 12:411-424. doi:10.1111/pbi. 12148
32. Bertani RP, Perera MF, Arias ME, Luque C, Funes C, González V, Cuenya MI, Ploper DL, Castagnaro AP (2014) A study of Sugarcane yellow leaf disease in Argentina. Plant Disease. doi.org/10.1094/PDIS-12-13-1251-RE 98: 1036-1042.
33. Filippone MP, Perera MF, Salgado M, García MG, Velicce GR, Castagnaro AP (2010) Diagnóstico molecular de enfermedades sistémicas de la caña de azúcar en Argentina: Ajuste metodológico y aplicaciones. Revista Industial Agrícola de Tucumán 87: 1-7.
34. Perera MF, Paz NV, Noguera AS, Filippone MP, Castagnaro AP (2012d) Diagnóstico molecular de enfermedades sistémicas en caña de azúcar. Revista Análisis de Semillas 21: 41-43.
35. Larkin PJ, Scowcroft WR (1981) Somaclonal variation - a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60: 197-214.
36. Perera MF, García MG, Noguera AS, Sepúlveda Tusek M, Filippone MP, Castagnaro AP (2010) Evaluación de la variación somaclonal en vitroplantas de caña de azúcar mediante marcadores moleculares. Revista Industial y Agrícola de Tucumán 87: 10-14.
37. Perera MF, Filippone MP, Noguera AS, Cuenya MI, Castagnaro AP (2012b) An overview of the sugarcane mosaic disease in South America". Functional Plant Science and Biotechnology 6 (Special Issue2): 98-107. (PrintISSN1749-0472).
38. Perera MF, Filippone MP, Ramallo CJ, Cuenya MI, García ML, et al. (2009) Genetic diversity among viruses associated with sugarcane mosaic disease in Tucumán, Argentina. Phytopathology 99: 38-49.
39. Yang ZN, Mirkov TE (1997) Sequence and Relationships of Sugarcane Mosaic and Sorghum Mosaic Virus Strains and Development of RT-PCR-Based RFLPs for Strain Discrimination. Phytopathology 87: 932-939.
40. Marie-Jeanne V, Ioos R, Peyre J, Alliot B, Signoret P (2000) Differentiation of Poaceae Potyviruses by reverse transcription polymerase chain reaction and restriction analysis. J Phytopathol 148: 141-151.
41. Würschmidt G, de Ramallo NEV, Levi C (1988) Distribución e incidencia de la roya de la caña de azúcar en Tucumán. Rev Avance Agroind 33: 5-8.
42. Funes C, Perez S, Bertani R (2015) Fist report of Puccinia kuehnii in Argentina. Plant Disease. In press.
43. Perera MF, Bertani RP, Arias ME, LaO Hechavarría M, Zardón Navarro MA, Debes MA, Luque AC, Welin B, Cuenya MI, Acevedo Rojas R, Ploper LD, Castagnaro AP (2015) Novel morphological features and molecular characterization of the causal agent of sugarcane orange rust. Plant Disease. Under review.
44. Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19: 6823-6831.
45. Dantur KI, Enrique R, Welin B, Castagnaro AP (2015) Isolation of cellulolytic bacteria from the intestine of *Diatraea saccharalis* larvae and evaluation of their capacity to degrade sugarcane biomass. AMB Express 5: 15.
46. Pardo EM (2015) Aproximaciones Biotecnológicas aplicadas al mejoramiento de la soja para la obtención de variedades con resistencia incrementada a estrés hídrico. PhD Thesis. National University of Tucumán, Argentina.
47. Ploper LD, González V, Gálvez MR, de Ramallo NV, Zamorano MA, García MG Castagnaro AP (2005) Detection of Soybean Rust caused by Phakopsora pachyrhizi in Northwestern Argentina. Plant Dis 89: 774. DOI: 10.1094/PD-89-0774B.
48. Rocha CML, Vellicce GR, García MG, Pardo EM, Racedo J, Perera MF, de Lucía AD, Gilli J, Bogado N, Bonecarrére V, German S, Marcelino FC, Ledesma F, Ploper LD, Welin B, Castagnaro AP (2015 Estimation of molecular diversity of Phakopsora pachyrhizi using AFLP markers. Electronic Journal of Biotechnology 18: 439-444.
49. Sendín LN (2012) Búsqueda de alternativas biotecnológicas sustentables para el manejo de las enfermedades de los cítricos. PhD Thesis. National University of Tucumán, Argentina.
50. Chiesa MA, Siciliano MF, Ornella L, Roeschlin RA, Favaro MA, et al. (2013) Characterization of a variant of *Xanthomonas citri* subsp. citri that triggers a host-specific defense response. Phytopathology 103: 555-564.
51. Orce IG, Sendín LN, Marano MR, Vojnov AA, Castagnaro AP, Filippone MP (2013) Novel set of real-time PCR primers for simultaneous detection of Liberibacter species associated with citrus Huanglongbing. Sci Agric 72: 252-259.

52. Pérez D, Mazzone L (2004) La actividad frutillera en la provincia de Tucumán y Argentina - Producción, exportaciones, importaciones, y precios de la frutilla fresca y congelada en Argentina. Publicación Especial n° 26. INTA-EEAOC. Tucumán, Argentina.

53. Ontivero M, Arias M, Ricci JD, Babot J, Albornoz P, Castagnaro AP (2000) Analysis of genetic similarities among species of Fragaria, Potentilla, and Duchesnea found in northwest Argentina by using morphological, anatomical, and molecular characters. Canadian Journal of Botany 78: 547-556.

54. García MG, Ontivero M, Díaz Ricci JC, Castagnaro AP (2002) Morphological traits and high resolution RAPD markers for the identification of the main strawberry varieties cultivated in Argentina. Plant Breeding 121: 76-80.

55. Howard CM, Maas JL, Chandler CK, Albregts EE (1992) Anthracnose of strawberry caused by the Colletotrichum complex in Florida. Plant Dis. 76: 976-981.

56. Racedo J, Salazar SM, Castagnaro, AP, Díaz Ricci JD (2013b) A strawberry disease caused by Acremonium strictum. Eur J Plant Pathol 137: 649-654.

57. Ontivero M, Zamora GM, Salazar S, Ricci JCD, Castagnaro AP (2011) Isolation of a strawberry gene fragment encoding an actin depolymerizing factor-like protein from genotypes resistant to Colletotrichum acutatum. Genome 54: 1041-1044.

58. García MG (2015) Identificación varietal y búsqueda de genes (segmentos de ADN) asociados y/o involucrados en la resistencia a Colletotrichum fragariae en frutilla. PhD Thesis. National University of Tucumán, Argentina.

59. Chalfoun NR, Grellet-Bournonville CF, Martínez-Zamora MG, Díaz-Perales A, Castagnaro AP, et al. (2013) Purification and characterization of AsES protein: a subtilisin secreted by Acremonium strictum is a novel plant defense elicitor. J Biol Chem 288: 14098-14113.

60. Castagnaro AP, Chalfoun NR, Racedo J, Salazar SM, Díaz Ricci JC. “Polipéptido que tiene actividad inductora de la defensa contra estrés biótico en plantas, secuencia de nucleótidos que lo codifica, microorganismo, composiciones y métodos”. INPI Nº Expte: P2011 01 00 854. Trámite 11042911. PCT/EP2012/070173. 2012/123614 A1.